Genetic variation in bile acid metabolism

Implications for lipoprotein homeostasis

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ABSTRACT

The objective of the studies described in this thesis was to investigate the impact of variation in genes involved in bile acid metabolism on lipoprotein homeostasis in humans. CYP7A1 is the rate-limiting enzyme in the catabolism of cholesterol into bile acids in the liver. We therefore hypothesized that the CYP7A1 A-278C polymorphism could have important consequences for lipid metabolism and we extensively studied the role of this polymorphism in different populations and on several endpoints.

First of all, we studied the role of the CYP7A1 A-278C polymorphism in four hyperlipidemic populations (patients with hypertriglyceridemia, combined hyperlipidemia, familial dysbetalipoproteinemia and familial hypercholesterolemia). We found no differences in the frequency distribution of the CYP7A1 A-278C alleles between these populations and a normolipidemic population; in all populations, approximately 40% of the subjects carried the C-allele. This indicates that the CYP7A1 A-278C polymorphism is not involved in the development of these disorders. However, when we investigated the association between the CYP7A1 A-278C polymorphism and serum lipids within each population, we did observe effects of the polymorphism. In the normolipidemic population, homozygous for the apoE3 isoform, we found an association between the AA genotype and higher levels of serum triglycerides (AA: +34%, p=0.036). Furthermore, in patients with hypertriglyceridemia the AA genotype was associated with significantly higher concentrations of total cholesterol (+23%, p<0.01).

Next, we hypothesized that the CYP7A1 A-278C polymorphism could be involved in the response of serum lipids to diet. We used data from 26 previously conducted dietary intervention trials and found that, upon an extra dietary cholesterol intake, subjects with the genotype CC had a higher response of serum HDL-cholesterol levels as compared to subjects with the genotype AA (0.17 ± 0.04 vs. 0.00 ± 0.02 mmol/l, resp; p<0.001). Furthermore, these subjects also displayed a higher response of serum total cholesterol upon intake of cafestol (1.01 ± 0.10 vs. 0.69 ± 0.10 mmol/l, resp; p=0.03). However, in a well-designed 12-week response study, we observed no significant differences in response of serum lipid levels between CYP7A1 A-278C genotype groups after a cholesterol-raising intervention (extra dietary cholesterol intake) and after a cholesterol-lowering intervention.
(cholestyramine intake). In this study, subjects were preselected for the CYP7A1 A-278C genotype and only AA and CC carriers participated. We therefore conclude that this polymorphism has no impact on serum lipid response. Furthermore, we observed no difference in plasma levels of 7alpha-hydroxy-4-cholesten-3-one between genotype groups, indicating that the CYP7A1 A-278C polymorphism does not result in differences in enzyme expression.

We also investigated the role of the CYP7A1 A-278C polymorphism in the progression of atherosclerosis and the risk of clinical events. We found that progression of atherosclerosis, as indicated by the mean segment diameter and minimum obstruction diameter in the coronary artery, was significantly lower in subjects with the genotype AA (33%, $p<0.01$ and 44%, $p=0.02$), resp.), as compared to subjects with the genotype CC. Inclusion of risk factors for CHD in the model showed the same trend, although not significant for MOD ($p=0.01$ for MSD and $p=0.06$ for MOD). Furthermore, the relative risk of a new clinical event was almost twice as high in subjects with the genotype CC (RR = 1.93; 95% CI 1.11-3.36; $p=0.02$). Inclusion of risk factors for CHD in the model showed the same trend, although not significant (RR=1.74; 95%CI 0.96-3.12; $p=0.06$). These data indicate a rather large impact of the CYP7A1 A-278C polymorphism on clinical development of atherosclerosis. However, before drawing firm conclusions, these results need to be replicated in another study.

Finally, we assessed the role of CYP7A1, CYP27 and IBAT, three important proteins in bile acid metabolism, in the heritable variation of serum lipid levels by means of linkage and association analyses in two independent Dutch twin populations. We observed no evidence for linkage of CYP7A1, CYP27 or IBAT to serum lipid levels (all LOD scores<1.0) indicating that polymorphisms in these genes will probably not affect serum lipid levels to a large extent.

Based on these studies, we conclude that the impact of genetic variants in genes important in bile acid metabolism on cholesterol homeostasis is very limited.
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GENERAL INTRODUCTION
BACKGROUND

An elevated level of cholesterol in blood is a major risk factor for the development and progression of cardiovascular disease. Therefore, over the past years, cholesterol metabolism has been one of the main focuses of scientific research. An important pathway for eliminating cholesterol from the body is via the bile, either directly or after conversion into bile acids. Therefore, it is of great interest to elucidate and to be able to modulate the bile acid synthetic pathway since this is a way to affect cholesterol levels beneficially. The aim of the studies in this thesis is to achieve a better understanding of genetic factors that are involved in bile acid and cholesterol metabolism in humans. This introduction gives a concise overview of cholesterol and bile acid metabolism and the role of genetic variations in these pathways. Based on this background, the rationale, research questions and the outline of this thesis are presented at the end of this chapter.

CHOLESTEROL METABOLISM

Cholesterol and triglycerides are the most predominant lipids in the circulation and play a role in many different cellular processes in the human body. The body obtains cholesterol and triglycerides via the diet and endogenous synthesis. Cholesterol is essential for the biosynthesis of cellular membranes, steroid hormones and bile acids. Triglycerides supply cells with fatty acids, which are used as an energy source in cardiac and skeletal muscle or for storage in adipose tissue. Cholesterol and triglycerides are both hydrophobic lipids and are therefore packaged into water-soluble particles, called lipoproteins, for their transport in the circulation.

The liver plays an important role in the regulation of serum cholesterol levels. Three metabolic pathways are integrated in the liver to maintain cholesterol homeostasis in the body (Figure 1.1): the exogenous pathway, the endogenous pathway and the reverse cholesterol pathway (1). The exogenous pathway concerns the transport of dietary cholesterol and triglycerides from the intestine to the liver. Dietary cholesterol and triglycerides are processed in the intestine and packaged into chylomicrons. Through the lymph, these chylomicrons enter the circulation. The capillary vessel wall of peripheral tissues contains lipoprotein lipase (LPL), which hydrolyzes the triglycerides in the core of the chylomicron into free fatty acids, which
subsequently enter fat and muscle cells to be either stored or used as an energy source. Due to the hydrolysis of the core lipids, the particle becomes smaller and is now called a chylomicron remnant. The chylomicron remnant, now relatively enriched in cholesterol, is rapidly cleared by the liver via apolipoprotein E (apoE) mediated binding to specific receptors, i.e. the low-density lipoprotein (LDL)-receptor and the LDL-receptor-related protein (LRP).

The metabolism of very low-density lipoproteins (VLDL), which delivers lipids from the liver to peripheral tissues, is called the endogenous pathway. Hepatic cholesterol and triglycerides are incorporated into nascent VLDL particles in the liver. Like chylomicrons, VLDL-triglycerides are hydrolyzed to deliver free fatty acids to the periphery, resulting in the formation of smaller VLDL-remnants, called intermediate density lipoproteins (IDL). IDL can either be cleared from the circulation by the liver, which is a process that is also mediated by apoE, or can be further hydrolyzed by hepatic lipase into LDL after a subsequent loss of triglycerides and apoE. LDL is then removed from the circulation by the LDL-receptor, using its apoB100 as a ligand.

The transport of cholesterol from peripheral tissues back to the liver is mediated by high-density lipoprotein (HDL) particles and is called the reverse cholesterol pathway. Nascent HDL appears in the circulation after synthesis by the liver and small intestine, and after lipolysis of chylomicrons. The ATP-binding cassette transporter A1 (ABCA1) actively transports cellular cholesterol to the outside of the cell, where nascent HDL (HDL-n) serves as a cholesterol acceptor. Cholesterol is then converted by lecithin/cholesterol acyltransferase (LCAT) into cholesteryl esters which accumulate in the HDL particle, which now becomes HDL3. Further uptake of cholesterol results in larger-sized, cholesterol-rich HDL2. HDL2 can then deliver cholesterol to the liver via three pathways. Firstly, cholesterol ester transport protein (CETP) can transfer cholesterol from HDL to VLDL, IDL and LDL in exchange for triglycerides. Secondly, after receiving apoE from other circulating lipoproteins, HDL can be cleared via hepatic apoE-mediated receptor uptake. Thirdly, cholesteryl esters can specifically be taken up from HDL by the scavenger receptor SR-B1. Cholesterol in the liver can subsequently be excreted from the body, either directly into bile or after conversion into bile acids.
FIGURE 1.1 Cholesterol homeostasis in the human body
BILE ACID METABOLISM

One of the major pathways for eliminating cholesterol from the body is via conversion into bile acids (2). There are two pathways involved in this catabolism of cholesterol (Figure 1.2). The classical pathway is initiated by the conversion of cholesterol into 7α-hydroxycholesterol, by the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (CYP7A1). The second conversion in this pathway is the formation of the metabolite 7α-hydroxy-4-cholesten-3-one, which can be measured in plasma and is used as a marker for monitoring CYP7A1 activity in vivo (3). The alternative pathway is initiated by the enzyme sterol 27-hydroxylase (CYP27). Finally, through these two pathways, the primary bile acids, cholate and chenodeoxycholate, are formed, which can be subsequently converted into deoxycholate and lithocholate by the intestinal flora.

The excretion of bile acids into bile is the driving force for the excretion of hepatic cholesterol into bile. Bile acids produced by the liver are secreted via the bile duct to the intestine. Here they play an important role in the solubilization and absorption of fats, fat-soluble vitamins and cholesterol. Then approximately 95% of the bile acids are actively transported back to the liver by, among others, the ileal bile acid transporter (IBAT/ASBT) (4). This cycling of bile acids between liver, gallbladder and intestine is called the enterohepatic circulation of bile acids and is essential for bile acid and cholesterol homeostasis.

In humans, the bile acid pool size is maintained relatively constant (range: 2500-4500 mg). Every day, the body is provided with, on average, 1000 mg cholesterol: 250 mg from diet and 750 mg from endogenous synthesis. Under normal circumstances, approximately the same amount of cholesterol will also be excreted from the body: 600 mg as free cholesterol and 400 mg as bile acids (5). In this way, the formation of bile acids accounts for a relatively high percentage to the daily elimination of cholesterol from the body.

In contrast to the efficient absorption of bile acids from the intestine, in humans, there is a large interindividual variability in the absorption of cholesterol from the intestine. Several studies indicate that cholesterol absorption rates range from 25%-75%, with an average of 50% (6,7). To be
absorbed, dietary sterols (cholesterol and plant sterols) must first be presented to the enterocyte brush border membrane in a solubilized form. Therefore, in the intestinal lumen, sterols are incorporated into mixed micelles, together with bile acids, fatty acids and phospholipids. The mechanism of uptake is not well understood. Recently, Niemann-Pick C1-like protein (NPC1L1), was identified as a critical protein for the intestinal absorption of cholesterol and plant sterols (8), but other proteins may also be involved. After uptake at the apical site, cholesterol and plant sterols can be excreted back into the intestinal lumen by ABCG5/ABCG8. Therefore, the net cholesterol absorption from the intestine is a balance between the transfer across the enterocyte brush border membrane and the excretion back into the intestinal lumen. Alternatively, cholesterol and plant sterols can be packaged into chylomicrons for their transport in the circulation at the basolateral site.

IMPACT OF BILE ACID METABOLISM

Several studies underscore the importance of bile acid synthetic capacity in cholesterol homeostasis. For example, a low bile acid synthetic capacity is an independent risk factor for the incidence of coronary heart disease and subnormal levels of bile acid synthesis are correlated to the progression of atherosclerosis and coronary mortality in patients with Familial Hypercholesterolemia (FH) (9,10). On the other hand, therapeutic compounds that increase bile acid synthesis (such as cholestyramine) have been shown to reduce serum LDL-cholesterol levels and thereby the risk of coronary heart disease (11). Interestingly, alterations in bile acid metabolism do not only affect cholesterol homeostasis but also triglyceride metabolism. For instance, patients with hypertriglyceridemia have an overproduction of bile acids (12). Individuals with a high synthesis rate of bile acids also display an increased rate of VLDL-triglyceride production by the liver (13). On the other hand, suppression of bile acid synthesis by feeding with chenodeoxycholate is linked to a reduced synthesis of VLDL-triglycerides (14,15). The mechanism behind this link between triglycerides and the rate of bile acid synthesis is unknown.
Regulation of CYP7A1

The expression of the rate-limiting enzyme in cholesterol catabolism CYP7A1, is tightly regulated by two nuclear receptors, the liver X receptor (LXR) and the farnesoid X receptor (FXR) (16,17). LXR is described as a cholesterol sensor and limits cholesterol accumulation in the body. Upon activation by oxysterols, LXR stimulates transcription of CYP7A1 and thereby the catabolism of cholesterol. Interestingly, the LXR-responsive element is not present in the human CYP7A1 gene, so the mechanism of action in humans is unknown. FXR is described as a bile acid sensor which protects the body from elevated bile acid concentrations. Bile acid activation of FXR represses the expression of CYP7A1 via increasing the expression of small heterodimer partner (SHP), a non-DNA binding protein. The increased abundance of SHP causes it to associate with the liver receptor homolog-1 (LRH-1), an obligate factor required for transcription of CYP7A1.

FIGURE 1.2 Bile acid metabolism in the human body
CHAPTER 1

ROLE OF DIETS

Different dietary modifications are known to affect serum cholesterol levels. For example, replacement of dietary carbohydrates by saturated fat increases serum LDL-cholesterol levels, leading to a higher risk of cardiovascular disease on a population level. On the other hand, replacement of carbohydrates for cis-unsaturated fat decreases serum LDL-cholesterol and increases HDL-cholesterol levels (18). Trans fat increases serum LDL-cholesterol levels, whereas HDL-cholesterol decreases (19). In a similar way, addition of cafestol, the cholesterol-raising factor in unfiltered coffee brews (e.g. espresso), increases serum LDL-cholesterol levels and slightly decreases serum HDL-cholesterol (20).

The precise mechanism determining the response of serum lipid levels to saturated and trans fatty acids is not known. Probably the number of hepatic LDL-receptors is involved (21). The response of serum lipid levels to dietary cholesterol is mediated through the release of sterol regulatory element-binding proteins (SREBP’s) (22). In sterol depleted cells, SREBP is cleaved by specific proteases and translocated to the nucleus. Here, SREBP binds to specific sites in the promoter region of certain genes, including those encoding HMG-CoA-reductase and the LDL-receptor. In cholesterol loaded cells, SREBP remains membrane bound and sterol synthesis and uptake from the circulation remain depressed. Recently, more insight was gained in the mechanism by which cafestol increases serum cholesterol levels (23,24). In mice, cafestol interacts with FXR and thereby causes a decrease in the expression of cholesterol 7α-hydroxylase and a subsequent reduction in fecal bile acid output. Whether the increase in serum LDL-cholesterol levels upon cafestol intake in humans is also caused by a decrease in CYP7A1 activity is not known.

Although elevated serum LDL-cholesterol levels in the general population result, in part, from the high intake of saturated fats and cholesterol, individuals vary widely in the response of their LDL-cholesterol concentrations to dietary fat and cholesterol (25). These variations are not explained solely by differences in compliance to following dietary guidelines, suggesting that genetic, hormonal or environmental factors are also involved in determining a person’s LDL-cholesterol level.
ROLE OF GENETICS

Data from family and twin studies indicate that genetic factors account for ~50% of the interindividual variation in LDL-cholesterol levels (26-29). A mutation in a gene can influence serum lipid levels by influencing the production and/or the function of the protein it encodes. There are many proteins and enzymes that play a role in maintaining cholesterol homeostasis. Since CYP7A1 is the rate-limiting enzyme in the catabolism of cholesterol, it seems plausible that genetic variants in CYP7A1 can have important consequences for cholesterol homeostasis.

Also animal studies have shown that there is an association between genetic differences in the synthesis of bile acids and the response of serum lipids to dietary interventions. Certain strains of mice, which are hypo-responsive to dietary fat and cholesterol by developing only mild elevated serum cholesterol levels, have a higher bile acid synthesis as compared to hyper-responders (30). Studies in which cholesterol 7α-hydroxylase was transiently over expressed in hamsters (31) and mice (32) showed a reduction in serum total- and LDL-cholesterol. Furthermore, cholesterol 7α-hydroxylase knockout mice have a strongly reduced bile acid synthesis, leading to a decreased VLDL production and a subsequent reduction in serum triglyceride levels (33). In pigs, polymorphisms in the cholesterol 7α-hydroxylase gene were associated with serum cholesterol levels (34).

Disruption of the CYP27 gene in mice leads to a reduced bile acid synthesis and bile acid pool size, despite a 5-fold increase in cholesterol 7α-hydroxylase activity (35). Furthermore, intestinal cholesterol absorption is decreased and fecal neutral sterol excretion is increased, leading to a compensatory increase in cholesterol synthesis in the liver (36). In addition, mice have markedly increased serum triglyceride levels. Interestingly, overexpression of the CYP27 gene in mice does not lead to changes in cholesterol and triglyceride levels, although biliary bile acid composition was slightly changed and fecal neutral sterols were slightly increased in females (37).

Mice in which the IBAT gene was disrupted show an increased bile acid and neutral sterol excretion in feces, and a consequent decrease in liver cholesteryl ester content (38). Furthermore, the expression of cholesterol 7α-hydroxylase was increased. Surprisingly, these changes did not affect serum LDL-cholesterol levels, but resulted in a slight increase in serum HDL-cholesterol levels.
CHAPTER 1

Polymorphisms in CYP7A1

Recently, a new monogenic disorder in humans was described: familial cholesterol 7α-hydroxylase deficiency (39). In patients with this disorder, of which, to date, only a few cases have been described, CYP7A1 activity was reduced by ~70%. Furthermore, patients display high levels of serum and hepatic cholesterol and a decrease in bile acid excretion from the feces. In addition, other patients suffered from hypertriglyceridemia and premature gallstone disease and their cholesterol levels were resistant to statin treatment. Besides this very rare CYP7A1 deficiency, also milder mutations or polymorphisms exist, which, on a population level, might influence cholesterol levels.

Wang et al. (40) reported that polymorphisms in the CYP7A1 gene account for 15% of the total variation in serum LDL-cholesterol in a sample of white Americans. This study also showed that the CYP7A1 locus is linked to high serum LDL-cholesterol levels, whereas no relationship was found between CYP7A1 and low serum LDL-cholesterol levels. DNA sequencing revealed a polymorphism located 278 base pairs upstream of the translation initiation codon at the CYP7A1 gene, referred to as the A-278C polymorphism (or A-204C in some studies). Approximately 15% of the general population carries the CC genotype of this polymorphism, 40% carries the AA genotype and 45% is heterozygous. Both in men and women, the CC genotype was associated with an increased serum LDL-cholesterol, as compared to the AA genotype. Since this study, several association studies on the A-278C polymorphism have been performed, however, with inconclusive results.

In line with the above described study, Hubacek et al. (41) reported that the CC genotype was associated with higher serum LDL-cholesterol levels compared to the AA genotype, however, only in a selected high cholesterolemic group of eighty-two children. In this study, no associations were found in the group of children with low cholesterol levels. In the Framingham Offspring Study (42), the CC genotype was associated with increased LDL-cholesterol levels in men and with decreased triglyceride levels in women. Furthermore, coronary heart disease was present in 17% and 6% in men and women, respectively, with the CC genotype, compared to 13% and 3% in the AA genotype group, which was significantly lower. However, these associations were no longer significant after adjustment for several covariates. In another study (43), the polymorphism was inconsistently associated with
serum lipid levels in three normolipidemic Canadian populations. In a group of 1100 individuals from the Pacific island of Kosrae, the CC genotype was associated with elevated levels of ApoA1 (44), which is associated with HDL.

As mentioned above, individuals vary widely in their response of LDL-cholesterol to dietary fat and cholesterol. Possibly, the CYP7A1 A-278C polymorphism not only plays a role in the establishment of serum lipid levels, but also influences the response of serum lipid levels to different diets and can thereby explain part of the observed interindividual differences in response. It was recently shown that after a lowering of dietary fat intake over a period of 8 years, subjects with the CC genotype have a significantly larger decrease in serum total cholesterol compared to subjects with the AA genotype (45). However, Friedlander et al. (46) found no effect of the CYP7A1 genotype on dietary responsiveness. Presently, no data are available on the effect of the CYP7A1 A-278C polymorphism on changes in serum lipids after dietary interventions that increase serum cholesterol.

The molecular mechanism underlying the effect of the CYP7A1 A-278C polymorphism on serum lipids is unknown, since no functional changes have been described for the A-278C mutation until now. Studies on the transcriptional regulation of CYP7A1 revealed that the promoter region between nucleotide -432 and -220 contains several cell-specific enhancer elements whose activity is controlled, in part, by Hepatocyte Nuclear Factor-3 (47). It is conceivable therefore, that the A-278C polymorphism might modulate transcriptional activity of the CYP7A1 gene and, consequently, the rate of cholesterol catabolism. However, it is also possible that the polymorphism in itself is non-functional, and is in complete linkage disequilibrium with another, functional, polymorphism in the CYP7A1 gene or in another unidentified gene nearby the CYP7A1 locus.

**Polymorphisms in CYP27 and IBAT**

Genetic variants in other important proteins in bile acid metabolism also stress the impact on cholesterol metabolism. For instance, loss of CYP27 in humans leads to cerebrotendinous xanthomatosis (CTX) (48), which is characterized by a striking deficiency in bile acids, a very low HDL-cholesterol and an accumulation of cholesterol in virtually all tissues. Furthermore, in patients with
Primary Bile Acid Malabsorption (PBAM), mutations in the bile acid transporter IBAT lead to severe interruption of the enterohepatic circulation of bile acids, diarrhea and reduced serum cholesterol levels (49). In vitro studies indicated that some of these mutations directly affected the transport of the bile acid taurocholate, whereas others were non-functional.

Although the above described mutations are rare and have relatively drastic consequences for cholesterol metabolism, it seems plausible that, as similar for the CYP7A1 gene, other genetic variants in these genes exist, which can have important, but milder impact on lipid metabolism.

**RATIONALE AND OUTLINE OF THIS THESIS**

This paragraph gives an overview of the following chapters in this thesis. The main goal of the studies described in this thesis was to gain more insight into the role of genetic variants in bile acid metabolism and the subsequent implications for lipoprotein metabolism. Several lines of evidence indicate the importance of mutations in genes involved in bile acid metabolism. The A-278C polymorphism in the CYP7A1 gene was found to be related to serum lipid levels. Furthermore, mutations in CYP27 and IBAT also lead to a disturbed lipid metabolism. Finally, also animal studies show the impact of genetic variation in bile acid metabolism on cholesterol homeostasis.

Based on these promising data in literature, we chose to extensively study the A-278C polymorphism in the CYP7A1 gene. Although previous studies were inconclusive about the precise role, we believed that this genetic variant could have important consequences for the homeostasis of serum cholesterol levels. Additionally, we chose to further explore the significance of genetic variation in CYP27 and IBAT in cholesterol metabolism.

We studied the role of the CYP7A1 A-278C polymorphism in various populations (normolipidemic and hyperlipidemic) and on different endpoints (serum lipids, response of serum lipids, clinical endpoints of atherosclerosis and clinical events) by means of association studies. Furthermore, we
conducted a response study in which subjects were preselected for the A-278C polymorphism. We also performed linkage analyses in two independent Dutch twin populations to find out the relevance of genetic variants in CYP27 and IBAT.

RESEARCH QUESTIONS

Is the A-278C polymorphism in the CYP7A1 gene associated with serum lipid levels in normolipidemic and hyperlipidemic individuals?

In chapter 2 we studied the association between the CYP7A1 A-278C polymorphism and serum lipid levels in a healthy normolipidemic population. Furthermore, we studied the role of this polymorphism in patients with several types of hyperlipidemic disorders: hypertriglyceridemia (HTG), combined hyperlipidemia (CH), familial dysbetalipoproteinemia (FD) and familial hypercholesterolemia (FH).

Is the A-278C polymorphism in the CYP7A1 gene associated with the response of serum lipid levels after dietary interventions?

In chapter 3 we performed a data analysis on the association between the A-278C polymorphism and the response of serum lipid levels after a period of excess dietary intake of saturated fat, trans fat, cholesterol and cafestol. To this end, data were pooled from 26 trials on diet and response, which were previously conducted at our Division. To further elucidate the role of the polymorphism and to confirm the results of the pooled data, we set up a response study, of which results are described in chapter 4.

Is the A-278C polymorphism in the CYP7A1 gene associated with the progression of atherosclerosis and the risk of new clinical events?

In chapter 5 we studied the association between the CYP7A1 A-278C polymorphism and two markers of atherosclerosis: the mean lumen diameter (mm) over the entire length of an obstructed vessel segment (MSD) and the lumen diameter (mm) of an obstructed segment at its most obstructed point (MOD), in patients included in the REGRESS study group. Furthermore, we studied whether the polymorphism is a risk factor for new clinical events.
CHAPTER 1

To what extent do CYP7A1, CYP27 and IBAT, three important genes in bile acid metabolism, contribute to heritable variation in serum lipid levels?

In chapter 6 we performed linkage analyses to assess the contribution of these three candidate genes to the heritable variation in serum lipid levels. For each gene, we selected three microsatellite markers in and around the gene and genotyped twin pairs and their parents for each marker. In this way, the co-inheritance of a specific phenotype (e.g. serum lipid levels) and a parental allele was investigated, resulting in a LOD-score (indicating linkage or not). In addition, we performed association analyses between the alleles of the microsatellite markers and serum lipid levels. With this study we could decide whether these 3 genes potentially contain sequence variants that can contribute to the heritable variation in serum cholesterol levels and whether it would be worthwhile to screen for new informative polymorphisms in these genes.

Finally, the main results of our studies are summarized in chapter 7. This chapter also focuses on the implications of our studies and the subsequent recommendations for future research.
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Modulating effect of the A-278C promoter polymorphism in the cholesterol 7α-hydroxylase gene on serum lipid levels in normolipidemic and hypertriglycerideremic individuals

Hofman MK, Groenendijk M, Verkuijlen PJJH, Jonkers IJAM, Mohrschladt MF, Smelt AHM, Princen HMG

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ABSTRACT

Background
The rate-limiting enzyme in the conversion of cholesterol into bile acids is cholesterol 7alpha-hydroxylase (CYP7A1). An A to C substitution 278 bp upstream in the promoter of the CYP7A1 gene was found to be associated with variations in serum lipid levels in normolipidemic populations.

Methods
In the present study we investigated the involvement of this polymorphism in four different lipid disorders: hypertriglyceridemia (HTG), combined hyperlipidemia (CH), familial dysbetalipoproteinemia (FD) and familial hypercholesterolemia (FH).

Results
In a normolipidemic male population, homozygous for the apoE3 isoform, an association was found between the genotype AA and higher levels of serum triglycerides (AA: +34%, \( p=0.036 \)). In HTG patients, the genotype AA was associated with significantly higher concentrations of total cholesterol (+23%, \( p=0.005 \)). There was a tendency towards increased levels of serum triglycerides (+39%, \( p=0.06 \)), VLDL-triglycerides (+48%, \( p=0.053 \)) and VLDL-cholesterol (+35%, \( p=0.059 \)). No significant associations were found between serum lipid levels and the CYP7A1 A-278C polymorphism in patients with CH, FD and FH.

Conclusion
Our results show that the A-278C polymorphism in the CYP7A1 gene has an effect on triglyceride levels in normolipidemic males and on cholesterol levels in patients with hypertriglyceridemia.
INTRODUCTION

It is well known that variation in serum lipid and lipoprotein levels is associated with the development and progression of atherosclerotic cardiovascular disease. Epidemiological studies demonstrate that about 50% of the variations in serum total and low density lipoprotein (LDL) cholesterol are caused by genetic factors (1-3). Polymorphisms in genes encoding proteins involved in cholesterol metabolism can therefore be important determinants for these interindividual differences. In addition, variations in such genes might also contribute to (or modulate) the development of disorders in which the lipid metabolism is disturbed.

The liver plays a central role both in the regulation and maintenance of the whole body sterol balance. Conversion of cholesterol into bile acids in the liver, together with secretion of cholesterol into bile is quantitatively the major pathway for eliminating cholesterol from the body (4). Several studies indicate that variations in the biosynthetic pathway of bile acids have important phenotypic consequences for both cholesterol and lipid metabolism (5-10).

The first and rate-limiting enzyme in the breakdown of cholesterol is cholesterol 7alpha-hydroxylase (CYP7A1) (4,11). An A to C conversion 278 bp upstream in the promoter has been found in the CYP7A1 gene (12). This polymorphism has been related to variations in serum lipid levels, however, results seem inconsistent. A significant increase in LDL-cholesterol was observed in homozygous C carriers, both in men and women (12). In the Framingham Offspring Study (13), in which more than 2000 subjects were studied, increased LDL-cholesterol levels were found, only in men. Furthermore, women homozygous for the C-allele had significantly lower triglyceride levels than heterozygotes (13). In another study, performed by Hegele et al. (14), the polymorphism was inconsistently associated with serum lipid levels in three normolipidemic Canadian populations. So far, the influence of this polymorphism has only been studied in normolipidemic populations.

Several common inherited lipid disorders are described in humans, however, the precise molecular background of most of these disorders is yet unknown. Patients with familial dysbetalipoproteinemia (FD or type III HLP) are characterized by elevated cholesterol and triglyceride levels due to the accumulation of chylomicron and very low density lipoprotein (VLDL) remnants in the serum. Another common metabolic disorder is Hypertriglycerideremia
(HTG), which is characterized by both overproduction and decreased clearance of triglyceride-enriched lipoproteins and by reduced concentrations of HDL-cholesterol. In combined hyperlipidemia (CH) multiple lipoprotein phenotypes are present in the individual or within a family, mostly elevated serum LDL- and VLDL-cholesterol levels. Because of the complex phenotypes of these diseases, they are thought to be heterogeneous multifactorial disorders, influenced by several genetic and environmental factors. In contrast, familial hypercholesterolemia (FH) is a common monogenetic disorder caused by mutations in the LDL-receptor gene, leading to elevated serum LDL-cholesterol concentrations, tendon xanthomas and premature development of coronary heart disease. Although the molecular mechanism underlying this defect is known, it is conceivable that additional genetic factors can contribute to the expression of the phenotype and thereby to the development and severity of this disorder.

Since these disorders are all characterized by disturbed serum lipid levels and CYP7A1 is a major regulator of sterol balance, but also may affect triglyceride levels (6), we investigated whether the CYP7A1 polymorphism is an additional genetic risk factor contributing to the expression or development of the four lipid disorders: HTG, FD, CH and FH.

METHODS

Subjects
In this study, we used five different groups of subjects. The first group consisted of healthy normolipidemic 35-year-old males (n = 290), randomly selected from three different geographic areas in the Netherlands (15). Furthermore, we used four groups of hyperlipidemic subjects. The first group were patients with endogenous HTG (n = 139). The diagnosis HTG was based on the means of two fasting blood samples obtained after a dietary period of at least 8 weeks. The diagnostic criteria for HTG were: serum triglycerides > 4.0 mmol/L, serum VLDL-cholesterol > 1.0 mmol/L and serum LDL-cholesterol < 4.5 mmol/L (16,17). Patients with FD (n = 157) were diagnosed on homozygosity for the apoE2 isoform (18). The FD population was divided into hypercholesterolemic and normocholesterolemic subjects. Hypercholesterolemic FD patients were defined as having total cholesterol
levels higher than the 90th percentile, whereas normocholesterolemic E2/E2 subjects had total cholesterol levels lower than the 90th percentile according to the age- and sex-related percentile levels of the Prospective Cardiovascular Munster Study (19). The diagnostic criteria for CH (n = 92) were total serum cholesterol > 7.5 mmol/L, serum triglyceride concentration > 2 mmol/L, VLDL-cholesterol > 1 mmol/L and absence of tendon xanthomas (18). Diagnosis of FH (n = 272) was based on the mean of two measurements of total serum cholesterol > 8.0 mmol/L, triglyceride levels < 2.5 mmol/L (or a VLDL-cholesterol level of lower than 1.0 mmol/L after ultracentrifugation) and a family history of hypercholesterolemia and/or premature cardiovascular disease or the presence of tendon xanthomas (18).

All serum lipid levels were measured before treatment with lipid-lowering drugs. Exclusion criteria for all disorders were secondary hyperlipidemia due to diabetes mellitus, renal, liver or thyroid disease, fasting glucose > 7.0 mmol/l and alcohol consumption > 40 g/day. Patients were not metabolically deranged. All subjects gave informed consent and the study protocol was approved by the ethical committee from the Leiden University Medical Centre, Leiden, The Netherlands.

Biochemical analyses

Venous blood samples were collected after an overnight fast. Serum was obtained after centrifugation at 1500 g for 15 minutes at room temperature. Total serum cholesterol and triglyceride levels were measured enzymatically, using commercially available kits (Boehringer, Mannheim, Germany). For determination of individual lipoproteins, three ml of fresh serum was ultracentrifuged for 15 hours at 232,000 g at 15°C in a TL-100 tabletop ultracentrifuge using a TLA-100.3 fixed angle rotor (Beckman, Palo Alto, CA, USA). The content of the ultracentrifuge tube was divided in a density (d) < 1.006 and d = 1.006 – 1.25 g mL-1 fraction, designated as the VLDL and LDL-HDL fraction, respectively. HDL-cholesterol was measured in the LDL-HDL fraction after precipitation of LDL with phosphotungstic acid and MgCl2. Insulin was measured with a conventional radio-immuno assay (Medgenix, Brussels, Belgium) and glucose with a Hitachi 747 analyzer according to standard procedures (Roche diagnostics, Mannheim, Germany). In the control population, apoB concentrations were measured by immunonephelometric assay (20) and serum apoA1 levels were quantified by radial immunodiffusion (21). ApoE phenotyping in the normolipidemic and the FD populations was
performed using a rapid micro-method based on isoelectric focusing of delipidated plasma followed by immunoblotting using polyclonal anti-apoE antiserum (22). For the FD population, the results were confirmed by apoE genotyping as previously described by Reymer et al. (23).

CYP7A1 A-278C promoter polymorphism
Genomic DNA was isolated from peripheral blood leucocytes by standard methods (24). The A to C substitution 278 bp upstream of the translation initiation codon was detected as described previously (12), however, the following alternative primers were used: 5’-TTG AGG GAT GTT AGG TGA GTA-3’ (sense) and 5’- AAG AAT AAG CCA TAG ACA AC-3’ (antisense), resulting in a 690 bp fragment. Amplification was performed for 35 cycles of 30 sec at 93°C, 30 sec at 55°C and 30 sec at 72°C with an initial denaturation period of 4 minutes. Reactions were performed in 25µl volumes containing 14.3 µl milliQ water, 100-200 ng genomic DNA, 0.2mM of each dNTP, 2.5 µl TAQ buffer, 0.5 U TAQ polymerase (HT Biotechnology Ltd., UK) and 5 pmol of each primer. The PCR-amplified DNA fragment was digested by the enzyme BSA I and fragments were resolved on a 2% agarose gel containing ethidium bromide.

Statistical analyses
For each population, the Hardy-Weinberg equilibrium was calculated by gene-counting and $\chi^2$ analysis. Genotype distributions between the different populations were tested with the $\chi^2$ test. Whenever serum lipid values showed no normal distribution, parameters were logarithmically transformed before analysis. Untransformed values are shown in the tables.

In the HTG and normolipidemic FD populations, the number of women was small (n=19 and n=18 respectively) and therefore man and women were combined. For the other populations, man and women were analyzed separately; however, if there were no differences in results, the data were combined. Differences in serum lipid levels among the CYP7A1 A-278C genotypes were tested with analysis of variance. For the hyperlipidemic populations, BMI, sex and age were taken as covariates. In case of significant differences, group means were compared by Fisher’s Least Significant Difference test for multiple comparisons. Differences were considered significant at $p<0.05$. 


RESULTS

Baseline characteristics of the patient groups and normolipidemic males are shown in Table 2.1. In comparison with the control group, either cholesterol, triglyceride or both levels were significantly elevated in the different patient groups, with the exception for the normolipidemic FD patients.

All individuals were genotyped for the CYP7A1 A-278C polymorphism. The genotype distributions of the CYP7A1 A-278C variants, as well as the observed frequencies of alleles, among the different populations, are shown in Table 2.2. The observed allele frequencies in all populations were in Hardy-Weinberg equilibrium. There were no significant differences in genotype distributions between the normolipidemic control population and the different hyperlipidemic disorders. Since all FD patients carry the E2 isoform of the apoE protein but only four percent will develop the hyperlipidemic phenotype, the FD population was divided into normolipidemic and hyperlipidemic subjects. However, no significant differences were found between genotype distributions of the normolipidemic and the hyperlipidemic FD patients. These data indicate that the CYP7A1 A-278C polymorphism plays no major role in the development of the four hyperlipidemic disorders.

Since no data were available for LDL- and HDL-cholesterol in the normolipidemic male population and apoB and apoA1 levels correlate well with LDL- and HDL-cholesterol levels, associations with these proteins (see Table 2.1) were determined. In the normolipidemic male population, no significant associations were found between serum lipid and apolipoprotein levels and the CYP7A1 A-278C genotype. However, there was a tendency towards increased triglyceride levels in subjects with the genotype AA as compared to genotype CC (+12%, p=0.074) (data not shown). Since the apoE isoform is known to influence serum lipid levels (15), the analysis was repeated with subjects only having the E3E3 isoform, the most predominant isoform (49% of this control group). When only E3E3 isoforms were included in the analysis, a 34% increase (p=0.036) in serum triglycerides was found in individuals homozygous for the A-allele as compared to individuals homozygous for the C-allele (Figure 2.1). No associations were found between the polymorphism and serum total cholesterol, apoB and apoA1 levels.
<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 290)</th>
<th>HTG (n = 139)</th>
<th>CH (n = 92)</th>
<th>FH (n = 272)</th>
<th>Total (n = 157)</th>
<th>Hyper (n = 109)</th>
<th>Normo (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n)</td>
<td>290</td>
<td>120</td>
<td>66</td>
<td>136</td>
<td>102</td>
<td>72</td>
<td>30</td>
</tr>
<tr>
<td>Female (n)</td>
<td>-</td>
<td>19</td>
<td>26</td>
<td>136</td>
<td>55</td>
<td>37</td>
<td>18</td>
</tr>
<tr>
<td>Age (y)</td>
<td>35 ± 10</td>
<td>48 ± 11</td>
<td>50 ± 11</td>
<td>47 ± 12</td>
<td>55 ± 15</td>
<td>50 ± 11</td>
<td>64 ± 17</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-</td>
<td>27.5 ± 3.2</td>
<td>26.7 ± 3.4</td>
<td>25.1 ± 3.5</td>
<td>26.4 ± 3.9</td>
<td>26.9 ± 3.9</td>
<td>25.2 ± 3.8</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>5.8 ± 1.1</td>
<td>8.7 ± 3.9</td>
<td>9.5 ± 1.9</td>
<td>9.8 ± 1.8</td>
<td>9.4 ± 3.9</td>
<td>11.1 ± 3.5</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.2 ± 1.4</td>
<td>14.2 ± 10.0</td>
<td>4.0 ± 2.1</td>
<td>1.8 ± 0.8</td>
<td>5.4 ± 4.6</td>
<td>6.7 ± 4.8</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>-</td>
<td>2.8 ± 1.5</td>
<td>6.8 ± 2.1</td>
<td>7.7 ± 1.9</td>
<td>3.5 ± 1.2</td>
<td>3.6 ± 1.2</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>VLDL-C (mmol/l)</td>
<td>-</td>
<td>4.9 ± 4.2</td>
<td>-</td>
<td>-</td>
<td>5.8 ± 4.1</td>
<td>6.3 ± 4.0</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>VLDL-TG (mmol/l)</td>
<td>-</td>
<td>11.8 ± 12.5</td>
<td>-</td>
<td>-</td>
<td>5.0 ± 4.8</td>
<td>5.5 ± 4.9</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>-</td>
<td>0.69 ± 0.20</td>
<td>1.00 ± 0.30</td>
<td>1.22 ± 0.34</td>
<td>1.12 ± 0.32</td>
<td>1.03 ± 0.28</td>
<td>1.35 ± 0.3</td>
</tr>
<tr>
<td>ApoB (mg/dl)</td>
<td>130 ± 42</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ApoA1 (mg/dl)</td>
<td>140 ± 23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- Values are mean ± SD
- Compared to the control group, both cholesterol and triglyceride levels were significantly elevated in the different patient groups, with the exception for the normolipidemic FD patients and triglyceride levels in the FH patients.
TABLE 2.2 CYP7A1 A-278C genotype and allele frequencies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>HTG</th>
<th>CH</th>
<th>FH</th>
<th>Total</th>
<th>Hyper</th>
<th>Normo</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>0.14 (42)</td>
<td>0.13 (18)</td>
<td>0.16 (16)</td>
<td>0.15 (42)</td>
<td>0.21 (33)</td>
<td>0.20 (22)</td>
<td>0.23 (11)</td>
</tr>
<tr>
<td>AC</td>
<td>0.48 (140)</td>
<td>0.45 (62)</td>
<td>0.43 (38)</td>
<td>0.45 (122)</td>
<td>0.45 (70)</td>
<td>0.45 (49)</td>
<td>0.44 (21)</td>
</tr>
<tr>
<td>AA</td>
<td>0.37 (108)</td>
<td>0.42 (59)</td>
<td>0.40 (38)</td>
<td>0.40 (108)</td>
<td>0.34 (54)</td>
<td>0.35 (38)</td>
<td>0.33 (16)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele</th>
<th>Controls</th>
<th>HTG</th>
<th>CH</th>
<th>FH</th>
<th>Total</th>
<th>Hyper</th>
<th>Normo</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.39</td>
<td>0.36</td>
<td>0.38</td>
<td>0.38</td>
<td>0.43</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>A</td>
<td>0.61</td>
<td>0.64</td>
<td>0.62</td>
<td>0.62</td>
<td>0.57</td>
<td>0.57</td>
<td>0.55</td>
</tr>
</tbody>
</table>

- No difference in frequency distribution of the polymorphism between hyperlipidemic patients and normolipidemic controls was found. Numbers between brackets indicate the actual number of subjects.
FIGURE 2.1 Effect of the CYP7A1 A-278C polymorphism on serum triglyceride levels in a normolipidemic male population.

Serum triglyceride levels according to CYP7A1 A-278C genotype are shown. Only carriers of the apoE3/E3 isoform were included in the analysis (n = 145).

To investigate the effect of the CYP7A1 A-278C polymorphism in the different lipid disorders, we divided the patients in homozygous A carriers and carriers of the C-allele. The groups of the genotype CC were too small for statistical analysis. HTG patients homozygous for the A-allele had significantly increased levels of total cholesterol (p=0.006), total triglycerides (p=0.039), VLDL-cholesterol (p=0.05) and VLDL-triglycerides (p=0.039) (data not shown). The inclusion of BMI, sex and age in the model slightly changed these results, as shown in Figure 2.2. In subjects homozygous for the A-allele, serum total cholesterol was significantly increased by 23% (p=0.005) as compared to carriers of the C-allele. There was a strong tendency towards increased levels of serum triglycerides (+39% p=0.06), VLDL-triglycerides (48% p=0.053) and VLDL-cholesterol (+35%, p=0.059) in homozygous A carriers. No associations between the CYP7A1 A-278C genotype and serum HDL- and LDL-cholesterol levels were found in HTG patients. Since insulin strongly influences serum VLDL levels in the HTG patients (25), we compared serum insulin and glucose concentrations and insulin resistance (HOMA-index) between the homozygous A carriers and the carriers of the C polymorphism. However, no significant differences were found for these parameters between the groups (data not shown).
Serum total cholesterol (TC), total triglycerides (TG), VLDL-cholesterol (VLDL-C) and VLDL-triglycerides (VLDL-TG) in HTG patients are shown divided by AA genotype (black bars) and AC/CC genotype (white bars). Values are adjusted for BMI, sex and age.

In patients with CH and FH and both in normolipidemic and hyperlipidemic FD patients, no significant associations were found between the CYP7A1 A-278C genotype and serum levels of total cholesterol, total triglycerides, LDL-cholesterol and HDL-cholesterol. Furthermore, no differences in results were found between men and women in these groups (data not shown).

DISCUSSION

In the present study we characterized the role of the A-278C promoter polymorphism in the cholesterol 7alpha-hydroxylase gene in a healthy normolipidemic male population and in four groups of patients with lipid disorders: HTG, CH, FH and FD. Allele frequencies of all studied populations in our study are comparable to those in literature (12); approximately 40% of the population carries the –278 C-allele. We did not find an effect on the frequency distribution of the CYP7A1 A-278C polymorphism between the
hyperlipidemic patients and the normolipidemic control population. This is a strong indicator that this variant does not contribute to the development of the hyperlipidemic disorders. Detailed association studies, however, showed a modulating effect of the polymorphism on serum lipid levels.

In a healthy normolipidemic male population, we found no significant associations between serum lipid levels and the CYP7A1 A-278C genotype. It is known, however, that the apolipoprotein E polymorphism is a modulator of serum cholesterol and triglyceride levels. Although results are variable, in several studies it was shown that the apoE polymorphism accounts for 2-20 percent of the interindividual differences in lipid and lipoprotein levels (26-28). For this reason we performed a second analysis in which we included only subjects homozygous for the E3 isoform, accounting for about 50% of the population. We found a significant 34% increase in serum total triglyceride levels in homozygous A carriers as compared to homozygous C carriers. Such a marked difference in triglyceride levels between homozygous A and C carriers has not been reported before, although Couture et al., described a small difference between CC and AC carriers in women only (13). We found no association with apoB levels, in agreement with other reports (13,14). An association of the CYP7A1 A-278C polymorphism has only been found with LDL-cholesterol levels, which were higher in carriers of the C-allele (12), although this has not been confirmed consistently in other populations (13,14). A reason for these differences may be differences in dietary intake and composition of the diet, which strongly affects serum lipid levels, and genetic background of the populations.

In HTG patients, we also found, for the first time, an association between the genotype AA and elevated levels of serum total cholesterol. Furthermore, there was a tendency towards increased levels of serum triglycerides, VLDL-triglycerides and VLDL-cholesterol.

Both in the healthy normolipidemic male population and in the HTG population, the polymorphism seems to be involved in triglyceride metabolism. We did not find this association in the other hyperlipidemic populations. For the FH population this association between the CYP7A1 A-278C polymorphism and serum triglycerides was not expected. It is known that FH is caused by a defect in the LDL-receptor gene, leading to an increase in serum LDL-cholesterol. This genetic defect does not lead to a change in serum triglyceride levels. For the hyperlipidemic FD population, an association between the CYP7A1 A-278C genotype and serum triglycerides could be expected. These
patients have high levels of triglycerides, which could partly be explained by the CYP7A1 A-278C genotype. The same is true for the CH patients. This disorder is associated with a complex phenotype, and probably multiple genetic defects play a role. However, we found no evidence for a role of CYP7A1 in these diseases.

The molecular mechanism underlying the effect of the CYP7A1 A-278C polymorphism on serum lipid levels is as yet unknown. Studies on the transcriptional regulation of CYP7A1 revealed that the promoter region between nt -432 and –220 contains several cell-specific enhancer elements whose activity is controlled, in part, by HNF-3 (29). It is conceivable therefore, that the A-278C polymorphism might modulate transcriptional activity of the CYP7A1 gene and, consequently, the rate of cholesterol catabolism. Theoretically, the association that we found between the genotype AA and elevated triglyceride levels in normolipidemic and HTG subjects could be explained by an increased bile acid synthesis in AA carriers. From previous studies it is known that there is a strong correlation between an increased bile acid synthesis and serum triglyceride levels (6). Treatment with the bile acid sequestrant cholestyramine, which induces bile acid synthesis, leads to an increase in VLDL-triglyceride and VLDL-cholesterol production. This increase is seen both in healthy individuals (30) and in patients with various types of hyperlipidemia (31), and predominantly in patients with HTG (32,33). In animals this relation between bile acid synthesis and serum triglyceride levels also exists. Disruption of the enzyme sterol 27-hydroxylase in mice, leads to a 5-fold increase in CYP7A1 activity and a 2-fold increase in hepatic and serum VLDL-triglyceride levels (5). For the HTG population this polymorphism could serve as an extra modulating genetic factor, increasing the triglycerides even more.

The hypothesis would also well be in line with previous research in which the genotype AA was found to be associated with decreased LDL-cholesterol levels in a normolipidemic population (12). If the bile acid synthesis is upregulated, more cholesterol is needed and will be acquired by the liver via LDL-receptor mediated uptake and, as a consequence, LDL-cholesterol in serum will be decreased. However, again it should be noted that this hypothesis is only based on association studies and therefore studies towards the functionality of the CYP7A1 A-278C mutation are required. Furthermore, we cannot exclude that our findings are based on chance. Another possibility is that the polymorphism in itself is non-functional, and is in complete linkage
disequilibrium with another, functional, polymorphism in the CYP7A1 gene or in another unidentified gene nearby the CYP7A1 locus.

In conclusion, these data show that the A-278C polymorphism in the CYP7A1 gene is associated with serum triglyceride levels in a normolipidemic male population and with serum cholesterol levels in HTG patients. There are no associations between the CYP7A1 A-278C polymorphism and serum lipid levels in patients with CH, FD and FH.

ACKNOWLEDGEMENT
This study was financially supported by a grant from ZonMw and the Netherlands Heart Foundation (NHS) (980-10-024). We thank Wim van Duyvenvoorde for excellent technical assistance.
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CHAPTER 2


CYP7A1 A-278C polymorphism affects the response of serum lipids after dietary cholesterol or cafestol interventions in humans

Hofman MK, Weggemans RM, Zock PL, Schouten EG, Katan MB, Princen HMG

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CHAPTER 3

ABSTRACT

Background
The response of serum lipids to dietary cholesterol and fat varies among individuals. Variations in genes involved in cholesterol metabolism can be important in these interindividual differences. The rate-limiting enzyme in the conversion of cholesterol into bile acids is cholesterol 7α-hydroxylase (CYP7A1).

Methods
We investigated the effect of the A-278C promoter polymorphism in the CYP7A1 gene on responses of serum lipids to an increased intake in dietary cholesterol (742 ± 114 mg/d), cafestol (57 ± 6 mg/d), saturated fat (change of 8-9 energy percent p/d) and trans fat (change of 10-11 energy percent p/d) in 496 normolipidemic subjects. These responses were measured in 26 previously published dietary trials.

Results
After adjustment for the apoE genotype effect, subjects with the genotype AA had a significantly lower increase of serum HDL-cholesterol to a cholesterol-rich diet than subjects with the genotype CC (0.00 ± 0.02 versus 0.17 ± 0.04 mmol/l; \( p < 0.001 \)). Upon intake of cafestol, subjects with the genotype AA had a significantly lower increase of serum total cholesterol than subjects with the genotype CC (0.69 ± 0.10 versus 1.01 ± 0.10 mmol/l; \( p = 0.028 \)). No effects of the CYP7A1 A-278C polymorphism were found in the saturated and trans fat interventions.

Conclusion
In conclusion, the CYP7A1 A-278C polymorphism has a small, but significant, effect on the increase of serum HDL-cholesterol and serum total cholesterol after an increased intake of dietary cholesterol and cafestol, respectively.
INTRODUCTION

The response of serum lipids to dietary cholesterol and fat varies among individuals, but within subjects this response is to some extent reproducible (1). Several studies in humans have shown that this response is associated with genetic polymorphisms (2). Variations in genes involved in cholesterol metabolism can therefore be important determinants for these interindividual differences and identification of genetic factors that affect the response can be helpful for selecting a proper treatment for hyperlipidemic patients.

The liver plays a central role in the regulation and maintenance of the whole body sterol balance. Conversion of cholesterol into bile acids in the liver, together with secretion of cholesterol into bile is quantitatively the major pathway for eliminating cholesterol from the body (3). In humans it was shown that changes in the biosynthetic pathway of bile acids lead to changes in cholesterol metabolism (4,5).

The first and rate-limiting enzyme in the catabolism of cholesterol is cholesterol 7α-hydroxylase (CYP7A1) (3). An A to C conversion 278 bp upstream in the promoter has been found in the CYP7A1 gene, which is related to variations in serum lipid levels (6). A significantly elevated LDL-cholesterol is observed in homozygous C subjects, both in men and women (6,7). Furthermore, women homozygous for the C-allele have significantly lower triglyceride levels than heterozygotes and than homozygotes for the A-allele (7).

Animal studies have shown that there is an association between genetic differences in the synthesis of bile acids and the response of serum lipids to dietary interventions (8-11). Since variations in cholesterol 7α-hydroxylase influence the cholesterol metabolism, polymorphisms in the CYP7A1 gene may be candidate genetic factors for the observed interindividual differences in response of serum lipids to a dietary challenge in humans. It was already shown that after a lowering of dietary fat intake, subjects with the CC genotype have a significantly larger decrease in serum total cholesterol (12). Presently, no data are available on the effect of the CYP7A1 A-278C polymorphism on changes in serum lipids after dietary interventions that increase serum cholesterol.

In the present study, we investigated the effect of the A-278C promoter polymorphism in the CYP7A1 gene on the response of serum lipids to changes
in intake of dietary cholesterol, saturated fat, trans fat and cafestol in 496 normolipidemic subjects.

METHODS

Subjects, diets and characteristics of the trials
Data from 26 controlled trials on diet and response of serum lipids, performed at the Division of Human Nutrition (Wageningen University, The Netherlands) have been pooled. Details about the design and methods of these trials are described in detail elsewhere (13). In short, in eight trials, the amount of dietary cholesterol was increased (1,14-17). The change in cholesterol intake was $742 \pm 114$ mg/d with a duration of 3-4 weeks. In seven trials saturated fatty acids were exchanged for an equal energy amount of cis-unsaturated fatty acids or carbohydrates (18-24). The change in intake was 8-9 energy percent with a duration of 3-4 weeks. In two trials trans fatty acids were exchanged for an equal energy amount of cis-unsaturated fatty acids with a change of intake of 10-11 percent and a duration of 3 weeks (22,23). In nine cafestol trials, subjects received coffee, coffee grounds, coffee oil or the coffee diterpenes cafestol and kahweol as a supplement without change in consumption in their habitual diet throughout the trial (25-30). The change in intake of cafestol was $57 \pm 6$ mg/d with a duration of 3-6 weeks. Cafestol is the substance that is responsible for the cholesterol-raising effect of unfiltered coffee.

All food was supplied in the seven trials with saturated fat, in the two trials with trans fat, and in three of the trials with dietary cholesterol. In the four trials of dietary cholesterol without complete food supply, subjects received eggs as a supplement during the treatment period and guidelines for a diet low in cholesterol during the control period. In one other trial of dietary cholesterol, subjects received all foods during the treatment period and received dietary guidelines during the control period.

We had data on the CYP7A1 A-278C and apoE genotype and total cholesterol levels for 104 subjects participating in the dietary cholesterol trials, for 112 subjects participating in the cafestol trials, for 201 subjects participating in the saturated fat trials, and for 79 subjects participating in the trans fat trials.
Because 41% of the subjects in the saturated fat trials and 56% of the subjects in the dietary cholesterol trials participated in more than one trial, we could calculate 209 and 291 responses of serum total cholesterol to dietary cholesterol and saturated fat, respectively. We had data on serum HDL-cholesterol levels for 88 subjects and data on triglyceride and LDL-cholesterol levels for 25 subjects participating in the dietary cholesterol trails (resulting in 179 and 51 responses, respectively). The precision of the estimation of responses of serum lipids to saturated fat and dietary cholesterol was high, because a large number of subjects participated in more than one trial with a similar treatment.

At the time of the trials, the subjects were healthy as indicated by a medical questionnaire and by absence of anemia, glucosuria and proteinuria. The protocols were approved by an Ethical Committee and informed consent was obtained from all subjects.

**CYP7A1 A-278C promoter polymorphism**

The CYP7A1 A-278C polymorphism was detected as previously described (31). More than 20% of all samples were performed in duplicate and showed similar results.

**Statistical analyses**

The response of serum lipids of one individual was calculated as the level of cholesterol at the end of the treatment that increased cholesterol minus the level of the same individual either 1) at the end of the treatment that lowered cholesterol, 2) the placebo treatment, 3) or the diet without the cafestol or cholesterol supplement (depending on the design of the trial).

We first checked whether there were differences between the 3 genotype groups in sex, age and body mass index, because these can be potential confounders of the relationship between the CYP7A1 A-278C genotype and serum lipid responses.

In the adjusted analysis, the response was corrected for several parameters. First, responses were adjusted for the apoE genotype effect, since the apoE genotype is known to influence the response of serum lipid levels (32). The responses were also adjusted for trial since there were background differences between the trials in diet, duration of the treatment and time of the year the trial
was performed. If a trial consisted of more than one treatment we created factors indicating each treatment within a trial. Furthermore, in the saturated fat and cholesterol trials, the responses were adjusted for subject, because 41% of the subjects in the saturated fat trials and 56% of the subjects in the dietary cholesterol trials participated in more than one trial with a similar treatment or in a crossover trial with three treatments (more details are given in (13)). In an additional analysis we adjusted for sex, age, BMI and change in weight, because these can be potential confounders of the relation between the CYP7A1 A-278C genotype and serum lipid response. We also calculated which percentage of the total variance of serum cholesterol could be explained by the CYP7A1 A-278C and apoE genotypes. This was calculated by dividing the Type III sum of squares of the specific genotype (corrected for the parameters described above) by the total corrected sum of squares.

Since the CC group is the smallest, the estimate of effects in this group is the least robust and the most open for potential confounding. Therefore, in an additional analysis, we combined AC and CC subjects and we looked at the difference in response between AA subjects and C-carriers. From previous studies it is known that genotype effects are different in men and women (13). For this reason we also analyzed genotype effects separately in men and women.

We tested the adjusted differences in response of serum lipids between subjects with the CYP7A1 A-278C genotypes by analysis of variance. In case of significant differences, group means were compared by Fisher’s Least Significant Difference test for multiple comparisons. Whenever serum lipid values showed no normal distribution, parameters were logarithmically transformed before analysis. Untransformed values are shown in the tables.

All analyses were performed with SAS statistical software. A \( p \)-value of <0.05 was considered significant. Values are presented as means ± SEM.

**RESULTS**

The CYP7A1 A-278C genotype distribution was as follows: 174 subjects were homozygous for the A-allele (35%), 84 subjects homozygous for the C-allele (17%) and 238 subjects were heterozygous (48%). The observed allele frequencies were in Hardy-Weinberg equilibrium.
There were no significant differences in baseline serum total cholesterol levels between the three CYP7A1 A-278C genotype groups in the different intervention groups. There were also no differences in sex, age and body mass index between the three CYP7A1 A-278C genotype groups.

Overall, an increased intake in dietary cholesterol resulted in an increase in serum total-, HDL- and LDL-cholesterol (all \( p<0.01 \)). In the cafestol and trans fat interventions there was an increase in serum total- and LDL-cholesterol and triglycerides (all \( p<0.01 \)). HDL-cholesterol decreased in these interventions (\( p=0.02 \) and \( p<0.01 \), respectively). In the saturated fat intervention, serum total-, HDL- and LDL-cholesterol increased (all \( p<0.01 \)). Serum triglycerides showed the same trend (\( p=0.07 \)). More detailed information on the responses to these interventions is given in reference (13).

**Dietary cholesterol intervention**

The adjusted response of serum HDL-cholesterol was significantly affected by the CYP7A1 A-278C genotype, being the lowest in subjects with the genotype AA (\( p<0.001 \)) (Table 3.1). When we analyzed men and women separately, results were the same in both groups (\( p=0.02 \) for men and \( p=0.02 \) for women) (data not shown). The effects on HDL-cholesterol and total cholesterol were even stronger when we looked at the effect of C-carriers (AC and CC combined) versus AA subjects (\( p=0.0001 \) and \( p=0.03 \) respectively). The responses were not materially affected by adjustment for sex, age, BMI and change in weight (data not shown).

The CYP7A1 A-278C polymorphism accounted for 3% of total variance in the response of serum total cholesterol (\( p=0.05 \)). Interestingly, the CYP7A1 A-278C polymorphism accounted for 20% of total variance in the response of serum LDL-cholesterol (\( p=0.002 \)). However, the apoE genotype did not contribute to the variance in response of serum total- and LDL-cholesterol after an increased dietary cholesterol intake.
TABLE 3.1 Effect of the CYP7A1 A-278C genotype on the response of serum lipid levels after dietary cholesterol and cafestol interventions in human

<table>
<thead>
<tr>
<th>Intervention</th>
<th>n</th>
<th>Response per CYP7A1 genotype</th>
<th>Significance overall</th>
<th>Significance AA vs C-allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA mmol/l</td>
<td>AC mmol/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>209</td>
<td>0.27 ± 0.07 (71)</td>
<td>0.43 ± 0.05 (118)</td>
<td>ns</td>
</tr>
<tr>
<td>HDL-C</td>
<td>179</td>
<td>0.00 ± 0.02 (61)</td>
<td>0.08 ± 0.01 (102)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG</td>
<td>51</td>
<td>-0.28 ± 0.19 (16)</td>
<td>-0.16 ± 0.12 (32)</td>
<td>ns</td>
</tr>
<tr>
<td>LDL-C</td>
<td>51</td>
<td>0.13 ± 0.14 (16)</td>
<td>0.38 ± 0.09 (32)</td>
<td>ns</td>
</tr>
<tr>
<td>Cafestol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>128</td>
<td>0.69 ± 0.10 (39)</td>
<td>0.90 ± 0.08 (58)</td>
<td>0.03</td>
</tr>
<tr>
<td>HDL-C</td>
<td>128</td>
<td>-0.08 ± 0.03 (39)</td>
<td>-0.05 ± 0.03 (58)</td>
<td>ns</td>
</tr>
<tr>
<td>TG</td>
<td>128</td>
<td>0.55 ± 0.08 (39)</td>
<td>0.64 ± 0.07 (58)</td>
<td>ns</td>
</tr>
<tr>
<td>LDL-C</td>
<td>128</td>
<td>0.53 ± 0.09 (39)</td>
<td>0.66 ± 0.07 (58)</td>
<td>ns</td>
</tr>
</tbody>
</table>

- Values represent means ± SEM (n)
- In the cholesterol and cafestol interventions, responses were adjusted for the apoE genotype effect, trial and subject
**Cafestol intervention**

Subjects homozygous for the A-allele had a significantly lower increase of serum total cholesterol as compared to homozygous C subjects ($p=0.028$) ([Table 3.1](#)). The lower response of AA subjects on total cholesterol was also clear when we looked at the effect of C-carriers (AC and CC combined) versus AA subjects ($p=0.04$). The responses were not materially affected by adjustment for sex, age, BMI and change in weight (data not shown).

The polymorphism accounted for 5 % ($p=0.07$) and 3 % ($p=0.07$) of total variation in the response of serum total cholesterol and LDL-cholesterol, respectively. However, the apoE genotype did not contribute to the variance in response of serum total cholesterol upon intake of cafestol.

**Saturated fat and trans fat interventions**

There were no differences in the response of serum lipid levels between the three CYP7A1 A-278C variants after an increase in saturated or trans fatty acids (data not shown).

**DISCUSSION**

CYP7A1 plays an important role in the catabolism of serum cholesterol. In the present study we investigated the effect of the A-278C promoter polymorphism in the CYP7A1 gene on the response of serum lipid levels to an increase in dietary intake of cholesterol, cafestol, saturated fat and trans fat. We found that the genotype CC is associated with a higher response of serum HDL-cholesterol and total cholesterol after an increase in dietary cholesterol and cafestol, respectively.

The role of polymorphisms in the CYP7A1 gene in relation with responses of serum lipid levels after cholesterol-raising diets has not been studied before. All responses were corrected for the apoE genotype effect, since Weggemans et al. (32) found, in the same data set as we used, that the apoE genotype affects the response of serum lipid levels to dietary interventions. In our analyses we chose not to adjust for baseline cholesterol levels. Baseline cholesterol may not be a confounder of the association between CYP7A1 and
cholesterol response as the cholesterol response may affect the cholesterol level instead of being affected by the level.

The molecular mechanism underlying the increased response of the CC-variant on serum cholesterol after an increase in dietary cholesterol and cafestol is as yet unknown. Hubacek et al. (12) also showed an increased response of the CC-variant after a lowering in dietary fat intake. Studies on the transcriptional regulation of CYP7A1 revealed that the promoter region between nt -432 and –220 contains several cell-specific enhancer elements whose activity is controlled, in part, by HNF-3 (33). It is conceivable therefore, that the A-278C polymorphism might modulate transcriptional activity of the CYP7A1 gene and, consequently, the rate of cholesterol catabolism. Theoretically, the increased response of the CC genotype can be explained by a decrease in bile acid synthesis. When bile acid synthesis decreases, less cholesterol is catabolized in the liver and as a consequence, serum cholesterol increases. The finding of an association between the genotype AA and a decreased LDL-cholesterol in a normolipidemic population by Wang et al. (6) would be in line with this contention. Also from large intervention studies it is known that there is a correlation between serum LDL-cholesterol and an increased bile acid synthesis (4). Serum LDL-cholesterol decreases when the bile acid synthesis in the liver is upregulated. Similarly, in gallstone patients, Reihner et al. (34) reported a decrease in LDL-cholesterol with an increase in CYP7A1 activity. However, this hypothesis has not yet been proven. It could be that the polymorphism in itself is non-functional, and is in complete linkage disequilibrium with another, functional, polymorphism in the CYP7A1 gene or in another unidentified gene nearby the CYP7A1 locus. Furthermore, we cannot exclude that part of our results are due to chance, since we analyzed several subgroups, which obviously increases the risk of chance associations. On the other hand, after subgroup analysis and adjustment for confounders all associations remained in the same direction.

In the cholesterol intervention study the higher response of serum cholesterol in the CC subjects is mainly caused by the larger increase in HDL-cholesterol in this group. Wang et al. (6) also found significant higher serum HDL-cholesterol levels in men homozygous for the C-allele. Furthermore, Machleder et al. (10) reported that cholesterol 7α-hydroxylase activity segregates with serum HDL-cholesterol concentrations in mice. However, for the moment, the relation between the CYP7A1 A-278C polymorphism and serum HDL-cholesterol remains unclear.
In this study we also found a link between the cholesterol response to cafestol and the CYP7A1 A-278C genotype. From previous studies it is known that cafestol leads to increased levels of total- and LDL-cholesterol in humans (27). We have shown that cafestol inhibits the bile acid synthesis in mice (35) and in rat hepatocytes (36) by down-regulation of CYP7A1 and CYP27 gene expression and inhibition of CYP7A1 enzyme activity. Suppression of bile acid synthesis in humans may also be an explanation for the increase in serum cholesterol levels. However, at this moment it is difficult to extrapolate our results physiologically.

Overall, we found mild effects of the CYP7A1 A-278C polymorphism. In the dietary cholesterol intervention, the polymorphism accounted for 3 and 20 % of the total variation in the response of serum cholesterol and LDL-cholesterol, respectively. However, these percentages might be overestimated since they are based on our population and our experimental conditions only. Interestingly, the apoE genotype did not contribute to the total variation in the response of serum cholesterol after dietary cholesterol and cafestol interventions. This lack of effect of the apoE genotype was already reported in two previous studies (6,12).

Several polymorphisms in other genes involved in lipid metabolism have been studied in relation with responses of serum lipid levels after dietary interventions. Effects have been reported for the enzymes LPL, hepatic lipase, LCAT and CETP (for review see reference (2)). However, the effects are small and often not reproducible. In addition, they account for only a small percentage of the total variance in dietary response of serum lipids. Therefore, it is unlikely that only one polymorphism is a major factor in determining the interindividual variation in response of serum lipid levels. Probably, several polymorphisms in different genes together with environmental factors are involved in this response.

In conclusion, the CYP7A1 A-278C polymorphism has an effect on the response of serum cholesterol after an increase in dietary cholesterol and cafestol, with a lower response in subjects homozygous for the A-allele. The polymorphism accounts for a small but significant proportion of the genetic variability in the response of serum lipid levels.
CHAPTER 3

ACKNOWLEDGEMENT

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REFERENCES


Serum lipid response to dietary cholesterol and cholestyramine; absence of modification by genetic variant in CYP7A1

Hofman MK, Kok FJ, Princen HMG, Schouten EG

Submitted for publication
BACKGROUND

Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the catabolism of cholesterol in the human body. Genetic variation in CYP7A1 might have a decisive impact on cholesterol response after dietary modifications and could thereby be used to identify subjects who will benefit from dietary guidelines.

METHODS

94 healthy subjects, preselected for the CYP7A1 A-278C polymorphism, participated in a 12-week response study. The study consisted of three periods of 4 weeks, during which subjects consumed a daily supplement. In period 1, they received a placebo powder, in period 2 they received 550 mg/d egg yolk powder (cholesterol-raising intervention) and in period 3 they received 8 g/d cholestyramine (cholesterol-lowering intervention).

RESULTS

Overall, the intake of extra cholesterol (550 mg/d) resulted in a significant increase in serum total cholesterol (5%), LDL-cholesterol (5%) and HDL-cholesterol (8%) levels. The intake of cholestyramine (8 g/d) resulted in a significant decrease in serum total cholesterol (12%) and LDL-cholesterol (21%) levels and in a significant increase in serum HDL-cholesterol (3%) and triglyceride (14%) levels. There were no significant differences in the response of serum lipids between the AA and CC genotypes of the CYP7A1 A-278C polymorphism to the cholesterol-raising and the cholesterol-lowering intervention. Furthermore, there were no significant differences in plasma 7α-hydroxy-4-cholesten-3-one between genotype groups.

CONCLUSION

The CYP7A1 A-278C polymorphism is not involved in the interindividual differences in serum lipid response to diet. This genetic variant therefore is not useful as a screening-tool to identify subjects who will benefit from dietary guidelines.
INTRODUCTION

An important strategy for reducing cardiovascular disease risk is the lowering of cholesterol levels in blood by dietary recommendations. However, the response of serum cholesterol to diet varies between individuals. Some subjects have a high response to changes in the intake of dietary cholesterol, whereas others do not respond at all (1). There are several candidate genetic factors that might influence this interindividual difference in response (2). Knowledge of these genetic factors can help to gain insight into the mechanisms by which diet affects serum lipid levels. Furthermore, it might help to predict whether individuals with high serum cholesterol levels will benefit from dietary guidelines or not.

The liver plays a central role in the regulation of cholesterol balance. Conversion of cholesterol into bile acids, together with secretion of cholesterol into bile is quantitatively the major pathway for eliminating cholesterol from the body (3). In humans, changes in the biosynthetic pathway of bile acids lead to changes in cholesterol metabolism. For instance, patients with familial cholesterol 7α-hydroxylase deficiency display high levels of serum and hepatic cholesterol levels and a decrease in bile acid excretion from the feces (4). On the other hand, therapeutic compounds that increase bile acid synthesis (such as cholestyramine) have been shown to reduce serum LDL-cholesterol levels and thereby the risk of coronary heart disease (5).

Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in the catabolism of cholesterol into bile acids. Genetic variants in this gene might therefore be important determinants for serum cholesterol levels. An A to C conversion 278 bp upstream in the promoter has been found, which is related to variations in serum lipid levels in normolipidemic individuals (6-8) and hypertriglyceridermic patients (9). Recently, we showed that this CYP7A1 A-278C polymorphism was also associated with variations in the response of serum lipid levels after an increased intake of dietary cholesterol and cafestol, but not after an increased fat intake (10). These data were obtained after combining and pooling several interventions studies that were carried out in the past at our Division. In another study, it was shown that the CYP7A1 A-278C polymorphism influences the change in serum lipid levels in response to population dietary change over a period of 8 years (11). In both studies, subjects with the genotype CC had the highest response of serum cholesterol levels to changes in diet.
Based on these previously reported studies we hypothesized that the CYP7A1 A-278C polymorphism plays an important role in the response of serum lipid levels to diet and could therefore be used as a screening-tool for individuals that might respond better to dietary recommendation than others. We studied the impact of the CYP7A1 A-278C polymorphism on the response of serum lipid levels after a cholesterol-raising and a cholesterol-lowering intervention, in a group of 94 healthy individuals with either the AA or CC genotype of this genetic variant. Since it is as yet unknown whether the CYP7A1 A-278C polymorphism leads to a functional change in the enzyme activity, we also investigated the functionality of the polymorphism by investigating plasma levels of 7α-hydroxy-4-cholesten-3-one, a marker that reflects CYP7A1 activity in the liver (12,13).

METHODS

Subjects
We recruited 415 subjects (aged 18-55 y, men and women) among employees and students of the Wageningen University and through advertisements in local newspapers and buildings of universities, sporting clubs and waiting rooms of physicians in or near the city of Wageningen, in June 2003. The aims and the protocol of the study were explained and all subjects gave their written informed consent. Subjects were screened for the CYP7A1 A-278C genotype. Furthermore, serum total cholesterol and triglyceride levels were assessed. Subjects were apparently healthy as assessed by a short medical questionnaire. Exclusion criteria were serum total cholesterol > 8.0 mmol/l, triglycerides > 3.0 mmol/l, heart-, stomach- or intestinal disease, a frequent loss of appetite or the use of medication known to affect blood lipids. From the 415 screened subjects, we selected 50 subjects with the genotype AA and 49 subjects with the genotype CC of the CYP7A1 A-278C polymorphism. At the beginning of the study, five subjects withdrew because of reasons unrelated to the study, leaving 94 subjects who completed the 12-week intervention study (45 AA and 49 CC). During the study, subjects were unaware of their own CYP7A1 A-278C genotype. All subjects received a financial reward. The Ethics Committee of the Wageningen University positively judged the study protocol.
Study design

The design of the study is shown in Figure 4.1. The screening took place approximately 4 weeks before the start of the 12-week intervention. Based on this screening, study subjects were selected (as described above). The 12-week study consisted of three periods of 4 weeks, during which subjects consumed a daily supplement on top of their habitual diet. The daily supplement consisted of 200 ml of orange juice (packaged 200 ml Appelsientje, Riedel, NL) and a powder. Subjects had to mix the orange juice with the powder themselves and drink this mixture directly after dinner every day.

During the run-in period (Period 1), egg-white powder was supplied (Blanken en Zn, Ede, The Netherlands). This period acted as a placebo-period in which subjects could habituate to drinking the supplement every day at the same point in time. During the second period (Period 2), egg-yolk powder was supplied (Blanken en Zn, Ede, The Netherlands). A daily amount of 20 gram was given, corresponding to 550 mg cholesterol. During the third period (Period 3), cholestyramine (8 g/d) was supplied (Bristol-Meyers Squibb, Woerden, The Netherlands).

FIGURE 4.1 Design of the study

<table>
<thead>
<tr>
<th>Week</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening</td>
<td>Period 1</td>
<td>Period 2</td>
<td>Period 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Run-in</td>
<td>Cholesterol 550 mg/d</td>
<td>Cholestyramine 8 g/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Vertical arrows indicate blood collection on different days, with a minimum of one day in between
At the end of each period, four blood samples were taken and subject's weight was measured. Furthermore, at the end of each period, the habitual energy intake of each subject was estimated by a food-frequency questionnaire (14). In this way we could assess whether the habitual dietary intake between the two genotype groups in the three different periods differed.

Throughout the entire study, subjects were urged to maintain their habitual diet and not to change their smoking habits, physical activities or vitamin and medicine intake. The participants kept diaries in which they recorded the intake of the supplement, any sign of illness, medication used, phase of menstrual cycle and any deviations from their diet and the protocol. According to these diaries, adherence to the study was excellent.

**Compliance**

Throughout the study we remained in regular contact (at least twice a week) with all subjects, by means of sending newsletters and informative messages about the trial and by telephone administered questionnaires about the progress and difficulties of the study. In this way we tried to increase awareness of the importance of the study and compliance. Every day, subjects marked the intake of the supplement in the diary. Furthermore, unconsumed supplements had to be returned. According to these diaries, 1%, 2% and 2% of the supplements was not consumed in period 1, period 2 and period 3, respectively.

**Blood collection and biochemical analyses**

At the end of each period, blood samples were taken after a 12 hour fasting period on days 22, 24, 26 and 29 of each period under standard conditions. We took four collections per period in order to reduce the within-subject standard deviation of serum cholesterol. Venipunctures were performed by the same persons, according to standard protocol, at identical location, time and weekdays, in identical body position. Serum was obtained by low speed centrifugation between 0.5 to 1 hour after venipuncture, stored at -80°C and analyzed enzymatically for total cholesterol, HDL-cholesterol and triglycerides. LDL-cholesterol levels were calculated using the Friedewald formula (15). All samples from one person were analyzed within the same run. Approximately 10% of all samples were analyzed in duplicate. The coefficient of variation
within runs was 1.1% for total cholesterol, 1.3% for HDL-cholesterol and 0.01% for triglycerides.

For the analysis of the bile acid precursor $7\alpha$-hydroxy-4-cholesten-3-one, we pooled plasma samples per subject for each period. We determined plasma levels of $7\alpha$-hydroxy-4-cholesten-3-one using the HPLC method as described by Gälman et al. (13). All samples from one person were analyzed within the same run. The within-run coefficient of variation was 8.0%.

**Genotyping**

The CYP7A1 A-278C polymorphism was detected using a Real-Time PCR approach with fluorescent Taqman probes. The probe specific for the A-allele was 5'-TTG AGA GAA CTT CAA C-3' and was labeled with the fluorescent label VIC. The probe specific for the C-allele was 5'-TTG AGA GAC CTT CAA C-3' and was labeled with the fluorescent label FAM. The following primers were used for amplification: 5'-GCA ATC AGA GAC CTG CAA TAC TTG-3' and 5'-CCA GGT CCG AAT GTT AAG TCA AC-3'. Reactions were performed in 25 µl volumes containing 6.5 µl water, 200 nM C- and A-allele probes, 900 nM primers and 12.5 µl Taqman Universal PCR Master Mix (Applied Biosystems). Amplification was performed for 35 cycles at 92°C for 15 sec as denaturation period followed by an annealing/extension period at 60°C for 1 min, with an initial denaturation period of 95°C of 10 min. The genotypes were determined on an ABI Prism 7700 instrument with use of the Sequence Detection System, version 1.7 (Applied Biosystems). This method was validated against a conventional measurement of the CYP7A1 A-278C polymorphism using PCR and subsequent restriction fragment analysis (6). About 10% of the samples were genotyped by both methods and all samples showed the same genotype measured with either method. The apoE genotype was determined as previously described (16).

**Statistical analyses**

A power calculation showed that 40 subjects per group would be sufficient to detect a significant difference ($p<0.05$) in the response of serum total cholesterol of 0.17 mmol/l (as estimated from reference (17)) between subjects with the AA and CC genotype, with a power of 80%.
The four values of serum lipids obtained from each subject at the end of each supplemental period were averaged and then used for the calculation of the individual responses in serum lipid levels between the different periods.

For the cholesterol-raising intervention, we defined the response of serum cholesterol to an increased intake of dietary cholesterol as the level of serum cholesterol in period 2 minus the level of cholesterol in period 1. For the cholesterol-lowering intervention, we defined the response of serum cholesterol to an intake of cholestyramine as the level of serum cholesterol in period 3 minus the level of cholestyramine in period 1.

Differences in response of serum lipid levels to dietary cholesterol and cholestyramine between the subjects with the AA and CC genotype were analyzed by a two-tailed Student's t-test for paired samples. We used the General Linear Models (GLM) procedure to adjust for potential confounders, such as sex, BMI, age and apoE genotype on differences in response between subjects with the AA and CC genotype. For the cholesterol-lowering intervention, we also included the level of serum cholesterol in period 2 in the model, since the response in period 3 might be dependent on the initial cholesterol level after period 2 (and there was no wash-out between the period 2 and 3). All analyses were performed with SPSS statistical software.

**RESULTS**

Baseline characteristics of the participants were similar for the two genotype groups (Table 4.1). Furthermore, there were no significant differences in the daily dietary food intake between the two genotype groups in the three periods (Table 4.2).

**Lipid response**

*Cholesterol-raising intervention*

As expected, an increased daily intake of 550 mg cholesterol for 4 weeks resulted in a significant increase of serum total cholesterol by 5%, of LDL-cholesterol by 5% and of HDL-cholesterol by 8% (Table 4.3). However, there was no significant difference in the response of serum total cholesterol, LDL-
cholesterol, HDL-cholesterol and triglycerides between the AA and the CC genotype groups. Adjustment for sex, age, BMI and apoE genotype did not materially affect the difference in response between the two genotype groups (data not shown).

*Cholesterol-lowering intervention*

As expected, the intake of 8 gram of cholestyramine for 4 weeks resulted in a significant decrease in serum total cholesterol by 12%, of LDL-cholesterol by 21% and an increase of serum triglycerides by 14% and HDL-cholesterol by 3% (*Table 4.4*). However, there was no significant difference in the response of serum total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides between the AA and the CC genotype groups. Adjustment for sex, age, BMI, apoE genotype and cholesterol levels at the end of period 2, did not materially affect the difference in response between the two genotype groups (data not shown).

*Functionality of the CYP7A1 A-278C polymorphism*

At the beginning of the study, there were no significant differences in plasma levels of 7α-hydroxy-4-cholesten-3-one between the two genotype groups.

*Cholesterol-raising intervention*

Plasma levels of 7α-hydroxy-4-cholesten-3-one were significantly increased by 18% upon intake of cholesterol (*p*=0.03) (*Table 4.3*). However, there was no significant difference in the response of 7α-hydroxy-4-cholesten-3-one levels between the two genotype groups. Plasma oxysterols are mostly found in the lipoprotein fractions. Therefore, a rise in plasma levels of 7α-hydroxy-4-cholesten-3-one could be explained by an increase in levels of lipoprotein fractions in plasma. Intake of cholesterol causes an increase in cholesterol in plasma. Therefore, it might be appropriate to divide the individual levels of 7α-hydroxy-4-cholesten-3-one by the corresponding level of total cholesterol (18). The mean increase of 7α-hydroxy-4-cholesten-3-one after this correction was 13% (*p*=0.10). There were no differences in the response of 7α-hydroxy-4-cholesten-3-one levels between the two genotype groups after this correction either.
Cholesterol-lowering intervention

Plasma levels of 7α-hydroxy-4-cholesten-3-one were significantly increased by 514% upon intake of cholestyramine ($p<0.01$) (Table 4.4). However, there was no significant difference in the response of 7α-hydroxy-4-cholesten-3-one levels between the two genotype groups. After correction for total cholesterol, the increase of 7α-hydroxy-4-cholesten-3-one was 606% ($p<0.01$). However, again, there were no differences in the response of 7α-hydroxy-4-cholesten-3-one levels between the two genotype groups after this correction.

**TABLE 4.1** Baseline characteristics of study subjects divided by CYP7A1 A-278C genotype

<table>
<thead>
<tr>
<th></th>
<th>CYP7A1 Genotype</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>CC</td>
<td></td>
</tr>
<tr>
<td>Men/women (n)</td>
<td>19</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>Age (y)</td>
<td>42</td>
<td>± 2</td>
<td>40</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>24.6</td>
<td>± 0.6</td>
<td>23.8</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>5.4</td>
<td>± 0.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.3</td>
<td>± 0.1</td>
<td>1.2</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.6</td>
<td>± 0.1</td>
<td>3.5</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.3</td>
<td>± 0.1</td>
<td>1.3</td>
</tr>
<tr>
<td>7α-hydroxy-4-cholesten-3-one (ng/ml)</td>
<td>6.6</td>
<td>± 0.9</td>
<td>6.8</td>
</tr>
<tr>
<td>ApoE genotype (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2/2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E2/3</td>
<td>6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>E3/3</td>
<td>23</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>E4 (E2/3/4)</td>
<td>16</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SEM
- E4 (E2/3/4) indicates genotype E2/4, E3/4 or E4/4
TABLE 4.2 Dietary intake of study subjects during the three study periods, divided by CYP7A1 A-278C genotype

<table>
<thead>
<tr>
<th></th>
<th>Period 1 Run-in</th>
<th>Period 2 Cholesterol</th>
<th>Period 3 Cholestyramine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td>Energy (kJ/d)</td>
<td>9197 ± 457</td>
<td>9050 ± 544</td>
<td>8439 ± 302</td>
</tr>
<tr>
<td>Fat (g/d)</td>
<td>86.1 ± 6.7</td>
<td>80.2 ± 6.1</td>
<td>72.7 ± 4.4</td>
</tr>
<tr>
<td>Saturated fat</td>
<td>33.8 ± 3.7</td>
<td>31.8 ± 2.8</td>
<td>27.2 ± 1.7</td>
</tr>
<tr>
<td>Monounsaturated fat</td>
<td>26.7 ± 2.1</td>
<td>24.3 ± 1.9</td>
<td>22.5 ± 1.6</td>
</tr>
<tr>
<td>Polyunsaturated fat</td>
<td>17.3 ± 1.1</td>
<td>16.2 ± 1.6</td>
<td>15.9 ± 1.6</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>177 ± 17</td>
<td>177 ± 18</td>
<td>152 ± 12</td>
</tr>
<tr>
<td>Carbohydrates (g/d)</td>
<td>259 ± 11</td>
<td>262 ± 18</td>
<td>249 ± 10</td>
</tr>
<tr>
<td>Alcohol (g/d)</td>
<td>10 ± 3</td>
<td>11 ± 2</td>
<td>10 ± 3</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SEM
## TABLE 4.3 Unadjusted response of serum lipid levels to 550 mg/d dietary cholesterol (Period 1 versus Period 2)

<table>
<thead>
<tr>
<th></th>
<th>Response to dietary cholesterol</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total response mmol/l ± SEM (%)</td>
<td>CYP7A1 AA mmol/l ± SEM (%)</td>
<td>CYP7A1 CC mmol/l ± SEM (%)</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>0.2 ± 0.0 (5)*</td>
<td>0.2 ± 0.0 (5)</td>
<td>0.2 ± 0.0 (4)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.0 ± 0.0 (-1)</td>
<td>0.0 ± 0.0 (-1)</td>
<td>-0.1 ± 0.0 (-1)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>0.14 ± 0.03 (5)*</td>
<td>0.16 ± 0.04 (5)</td>
<td>0.12 ± 0.04 (4)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.10 ± 0.03 (8)*</td>
<td>0.09 ± 0.02 (7)</td>
<td>0.11 ± 0.01 (9)</td>
</tr>
<tr>
<td>7α-hydroxy-4-cholesten-3-one (ng/ml)</td>
<td>0.67 ± 0.47 (18)*</td>
<td>0.84 ± 0.54 (23)</td>
<td>0.52 ± 0.75 (13)</td>
</tr>
<tr>
<td>Corrected 7α-hydroxy-4-cholesten-3-one (mg/mol)</td>
<td>0.11 ± 0.07 (13)</td>
<td>0.09 ± 0.09 (18)</td>
<td>0.14 ± 0.10 (8)</td>
</tr>
</tbody>
</table>

- Values are responses ± SEM, with percentage of response between brackets
- Corrected 7α-hydroxy-4-cholesten-3-one indicates the ratio of 7α-hydroxy-4-cholesten-3-one/total cholesterol (mg/mol)
- * indicates $p<0.01$
<table>
<thead>
<tr>
<th>Table 4.4</th>
<th><strong>Unadjusted response of serum lipid levels to 8 g/d cholestyramine (Period 1 versus Period 3)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Response to cholestyramine</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Total response mmol/l ± SEM (%)</strong></td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>-0.7 ± 0.1 (-12)*</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.2 ± 0.0 (14)*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>-0.78 ± 0.05 (-21)*</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.04 ± 0.01 (3)*</td>
</tr>
<tr>
<td>$7\alpha$-hydroxy-4-cholesten-3-one (ng/ml)</td>
<td>26.7 ± 2.2 (514)*</td>
</tr>
<tr>
<td>Corrected $7\alpha$-hydroxy-4-cholesten-3-one (mg/mol)</td>
<td>5.7 ± 0.4 (606)*</td>
</tr>
</tbody>
</table>

- Values are responses ± SEM, with percentage of response between brackets.
- Corrected $7\alpha$-hydroxy-4-cholesten-3-one indicates the ratio of $7\alpha$-hydroxy-4-cholesten-3-one/total cholesterol (mg/mol).
- * indicates $p<0.01$.
CYP7A1 is the rate-limiting enzyme in the catabolism of cholesterol in the human body. On a population level, genetic factors in CYP7A1 have been shown to influence cholesterol homeostasis. In this study we hypothesized that a polymorphism in CYP7A1 could have a decisive impact on cholesterol response after diet, and could thereby be used as an indicator for subjects who will benefit from dietary changes. However, we found that the CYP7A1 A-278C polymorphism did not affect the response of serum lipid levels after an increased intake of dietary cholesterol. Furthermore, the polymorphism did not affect the response of serum lipid levels after a cholesterol-lowering intervention, in which the bile acid synthesis was stimulated using cholestyramine. In addition, levels of \(7\alpha\)-hydroxy-4-cholesten-3-one in plasma did not differ between the two genotype groups, suggesting that this polymorphism does not lead to a functional change in enzyme activity.

The 5\% increase in serum total cholesterol to a daily extra intake of 550 mg cholesterol was in line with responses observed in literature (19). Clearly, a daily bolus of cholesterol for several weeks, in a free-living situation, is sufficient to mildly increase serum cholesterol levels. In our previous study we found that subjects with the genotype CC had a higher response to an increase in dietary cholesterol intake than subjects with the genotype AA (20). However, these results were obtained after combining eight different intervention studies that were conducted in the past at our Division, and we could not exclude that the pooling of data from studies involving different interventions might have influenced the results. Therefore, one of the aims of the present study was to reproduce these results in a study design which was specifically set up to examine the role of the CYP7A1 A-278C polymorphism. Participants were preselected for the CYP7A1 A-278C polymorphism to ensure adequate statistical power. However, we could not confirm earlier results in the present study, and therefore we can conclude that this genetic variant does not play a role in the response of serum lipid levels to an intake of extra dietary cholesterol.

As expected, the intake of cholestyramine resulted in a marked decrease of serum total- and LDL-cholesterol levels. Cholestyramine binds to bile acids in the intestine and thereby inhibits their re-uptake and transport back to the liver. As a consequence, bile acid synthesis is increased, which causes an enhanced demand of cholesterol in the liver. The expression of the LDL-
CYP7A1 AND LIPID RESPONSE TO DIET (2)

receptor is increased, causing an increased uptake of cholesterol from serum. Administering 16 grams of cholestyramine per day to gallstone patients over a period of 2.5 weeks was shown to increase the activity of CYP7A1 about 6-fold (21). The 5-fold increase of 7alpha-hydroxy-4-cholesten-3-one levels in plasma in our study also indicates an increased activity of CYP7A1 and is in line with these data. If the CYP7A1 A-278C polymorphism would affect enzyme activity, we hypothesized that, upon such a large stimulation of the enzyme by cholestyramine, the differences between the two genotype groups would have been apparent even more, resulting in a clear difference in response between the two groups. However, we found no differences in response of serum lipid levels between the two genotype groups. In an observational study, Hubacek et al. (11) found a larger decrease in serum total cholesterol levels in subjects with the genotype CC over a period of 8-years, in a group of men in whom serum cholesterol levels markedly decreased. However, this reduction in serum cholesterol levels was due to a change in dietary composition and was not, as in our study, drug induced.

In literature, several association studies have been described with the CYP7A1 A-278C polymorphism and its effect on serum lipid levels. However, up till now, no studies have focused on the functionality of this genetic variant. It is ethically not acceptable to take liver biopsies from individuals to study CYP7A1 activity in humans and therefore, in the present study, we decided to use an indirect method. If the polymorphism would lead to a functional change in enzyme activity, we hypothesized that this might be reflected by the amount of 7α-hydroxy-4-cholesten-3-one in plasma, since this metabolite was shown to be a marker of bile acid synthesis in humans (12,13). The increase in plasma levels of 7α-hydroxy-4-cholesten-3-one upon intake of extra cholesterol and cholestyramine clearly demonstrated that CYP7A1 enzyme activity was indeed upregulated in our population. In addition, the large increase of 7α-hydroxy-4-cholesten-3-one in plasma upon intake of cholestyramine had been previously reported (22). However, in the present study we found no difference in the amount of 7α-hydroxy-4-cholesten-3-one in plasma between the two CYP7A1 A-278C genotype groups. Therefore, our results do not support a functional role for the CYP7A1 A-278C polymorphism.

In the past years, several studies have focused on the impact of genetic factors on the response to diet, since knowledge of these genetic factors can be useful in clinical and public health settings to find the best dietary advice for an individual. The best-studied candidate gene is that coding for apoE. It was
shown that a low-fat low-cholesterol strategy may be particularly beneficial in lowering serum cholesterol levels in subjects carrying the apoE4 allele of the apoE genotype (23). Correction for the apoE genotype effect did not change our results. Furthermore, our data indicate that the CYP7A1 A-278C polymorphism will not be helpful in identifying interindividual differences in response to diet.

In conclusion, the CYP7A1 A-278C polymorphism did not affect the response of serum lipids after a cholesterol-raising and a cholesterol-lowering intervention. We therefore conclude that, in medical practice, this variant cannot be used as a screening-tool to identify subjects who will benefit from dietary guidelines.

ACKNOWLEDGEMENT

This study was financially supported by a grant from ZonMw and the Netherlands Heart Foundation (NHS) (980-10-024). We thank Rien Buytenhek for excellent technical assistance with HPLC measurements and Karin Borgonjen for excellent dietary advices and practical help during the study.
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20 Hofman MK, Weggemans RM, Zock PL, Schouten EG, Katan MB, and Princen HM. CYP7A1 A-278C Polymorphism Affects the Response of Plasma Lipids after


Genetic variation in the rate-limiting enzyme in cholesterol catabolism (cholesterol 7alpha-hydroxylase) influences the progression of atherosclerosis and risk of new clinical events

Hofman MK, Princen HMG, Zwinderman AH, Jukema JW

Clinical Science

2005. In press
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ABSTRACT

Background
Coronary Heart Disease (CHD) is a complex disorder which is, in part, related to serum cholesterol levels. The rate-limiting enzyme in the catabolism of cholesterol into bile acids is cholesterol 7alpha-hydroxylase (CYP7A1).

Methods
The effect of the A-278C promoter polymorphism in the CYP7A1 gene on the progression of atherosclerosis, risk of a new clinical event and the influence of this variant on cholesterol-lowering therapy was investigated in 715 male patients with coronary atherosclerosis, participating in the REGRESS-study.

Results
CYP7A1 A-278C genotype distribution was as follows: 283 AA, 330 AC and 102 CC. There were no significant differences in baseline characteristics and serum lipid levels between the three genotypes. After two years, CC-carriers had significantly more progression of diffuse and focal atherosclerosis as compared to AA-carriers, as indicated by a larger decrease in both the Mean Segment Diameter (resp. 0.09 mm versus 0.06 mm; \( p=0.009 \)) and the Minimum Obstruction Diameter (resp. 0.09 mm versus 0.05 mm; \( p=0.024 \)) in the CC group. Inclusion of risk factors for CHD in the model showed the same trend, although not significant for MOD (\( p=0.01 \) for MSD and \( p=0.06 \) for MOD).

In addition, CC-carriers had an almost two-fold higher risk of a new clinical event as compared to AA-carriers (RR=1.93; 95%CI 1.11-3.36; \( p=0.02 \)). Inclusion of risk factors for CHD in the model showed the same trend, although not significant (RR=1.74; 95%CI 0.96-3.12; \( p=0.06 \)).

Conclusion
In conclusion, we present evidence that the CC variant of the A-278C polymorphism in the rate-limiting enzyme in the catabolism of cholesterol, CYP7A1, increases the progression of atherosclerosis and the risk of a new clinical event.
INTRODUCTION

Coronary Heart Disease (CHD) is a complex condition that is influenced by numerous environmental and genetic factors (1). Elevated levels of total- and LDL-cholesterol are thought to be primary risk factors for development and progression of CHD. Polymorphisms in genes encoding proteins involved in cholesterol metabolism can therefore influence the development and progression of CHD.

The liver plays a central role in the regulation and maintenance of the whole body sterol balance. Conversion of cholesterol into bile acids in the liver, together with secretion of cholesterol into bile is quantitatively the major pathway for eliminating cholesterol from the body. The first and rate-limiting enzyme in the breakdown of cholesterol is cholesterol 7alpha-hydroxylase (CYP7A1) (2,3). An A to C conversion 278 bp upstream in the promoter has been found in the CYP7A1 gene. Several studies have investigated the association between this CYP7A1 A-278C polymorphism and baseline serum lipid levels. A significantly elevated LDL-cholesterol has been observed in homozygous C carriers, both in men and women (4). Furthermore, women homozygous for the C-allele were demonstrated to have significantly lower triglyceride levels than heterozygotes (5). Couture et al. (5), investigated the effect of this polymorphism on the risk of CHD and found that both men and women with the genotype CC have a more frequent history of CHD as compared to AA subjects. However, after adjustment for several baseline parameters (e.g. age, BMI, smoking), the association between the CYP7A1 A-278C genotype and CHD was no longer significant. Effects of this polymorphism on progression of atherosclerosis and other parameters related to atherosclerosis have not been studied before.

The primary objective of the present study was to investigate the effect of the A-278C polymorphism in the CYP7A1 gene on 1) serum lipid levels, 2) progression of atherosclerosis, 3) risk of a new clinical event and 4) the influence of this variant on efficacy of cholesterol-lowering therapy using data from the REGRESS-study. The REGRESS-study is a double-blind, placebo controlled study to assess the effects of 2 years of treatment with HMG-CoA reductase inhibitor pravastatin on progression and regression of angiographically documented coronary atherosclerosis in male patients with serum cholesterol between 4 and 8 mmol/l (6).
METHODS

Subjects and study design

Detailed descriptions on the design and methodology of the REGRESS-study have been described elsewhere (6). In short, the REGRESS-study is a double-blind, placebo-controlled, multicenter study to assess the effect of 2-year treatment with pravastatin on the progression and regression of angiographically documented coronary atherosclerosis in 885 male patients undergoing coronary cinearteriography to assess anginal complaints. The patients were < 70 years old and had normal to moderately increased serum cholesterol levels (between 4.0 - 8.0 mmol/l), serum triglyceride concentrations of less than 4 mmol/l and at least one coronary artery stenosis causing more than 50% diameter reduction. All patients were of Caucasian descent. Baseline and follow-up coronary arteriograms were analyzed by quantitative computer analysis. Patients were randomly assigned to statin or placebo treatment, after stratification for hospital (n=11) and initial treatment (medical management, percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass graft surgery (CABG)).

A number of substudies have been performed in addition to the angiographic main study. Substudies included B-mode ultrasound studies of the carotid and femoral arteries, extensive lipid as well as other biochemical parameter research, and DNA studies among others (7-9).

The study was conducted under the auspices of the Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht, The Netherlands. Written informed consent was obtained from all patients, and the study was performed in accordance with the Declaration of Helsinki.

Of the 885 patients that participated in the REGRESS-study, from 715 patients DNA was available and they were successfully genotyped for the CYP7A1 A-278C polymorphism. Baseline serum lipid levels, as well as serum lipid levels after pravastatin or placebo treatment were available. Information about smoking status and family history of CHD was available from the case record form. The family history was considered positive if one of the parents had a myocardial infarction before the age of 60.

The angiographic endpoints in this study were defined before the study was unblinded. The primary endpoint was a comparison between the pravastatin and placebo groups for 1) change in average Mean Segment Diameter (MSD)
on a by patient basis and 2) change in average Minimum Obstruction Diameter (MOD) on a by patient basis. The MSD is the mean lumen diameter (in mm) over the entire length of an obstructed vessel segment. The MOD is the lumen diameter (mm) of an obstructed segment at its most obstructed point. The average MSD, reflecting diffuse changes of atherosclerosis, and the average MOD, reflecting focal changes of atherosclerosis, were calculated from the coronary angiograms at baseline and after 24 months. A larger decrease in the MSD and MOD reflects more progression of coronary atherosclerosis.

Another endpoint in this study was the occurrence of new clinical events. These included myocardial infarction (MI), nonscheduled PTCA, nonscheduled CABG, stroke, transient ischemic attack (TIA) and death, assessed prospectively by an independent clinical event committee.

**Serum lipids**

Serum total- HDL- and LDL-cholesterol and triglycerides were measured on fasting blood samples by standard techniques as described previously (6). In 256 patients, subclasses of LDL were analyzed by gradient gel electrophoresis (GGE) and classified according to Austin *et al.* (10). Class A = predominantly large buoyant LDL; Class B = predominantly small dense LDL; Class A/B = no distinctive A- or B- pattern present.

**CYP7A1 A-278C promoter polymorphism**

The A to C substitution 278 bp upstream of the translation initiation codon was detected using a Real-Time PCR approach with fluorescent TaqMan probes. The probe specific for the A-allele was 5’-TTG AGA GAA CTT CAA C-3’ and was labeled with the fluorescent label VIC. The probe specific for the C-allele was 5’-TTG AGA GAC CTT CAA C-3’ and was labeled with the fluorescent label FAM. The following primers were used for amplification: 5’-GCA ATC AGA GAC CTG CAA TAC TTG-3’ and 5’-CCA GGT CCG AAT GTT AAG TCA AC-3’. Reactions were performed in 25 µl volumes containing 6.5 µl water, 200 nM C- and A-allele probes, 900 nM primers and 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems). Amplification was performed for 35 cycles at 92°C for 15 sec as denaturation step followed by an annealing/extension period at 60°C for 1 min, with an initial denaturation period of 95°C of 10 min. The genotypes were determined on an ABI Prism 7700 instrument with use of the Sequence Detection System, version 1.7
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(Applied Biosystems). This method was validated against a conventional measurement of the CYP7A1 A-278C polymorphism using PCR and subsequent restriction fragment analysis (4). About 10% of the samples were genotyped by both methods and all samples showed the same genotype measured with either method.

Statistical analyses

Differences in baseline characteristics and concentrations of serum lipids among the CYP7A1 A-278C genotypes were tested by one-way analysis-of-variance (ANOVA) or Pearson’s chi-square test for frequencies. Differences in serum lipids among the CYP7A1 A-278C genotypes were adjusted for age and BMI. The Hardy-Weinberg equilibrium was calculated by gene-counting and $\chi^2$ analysis.

Changes in serum lipid levels and MSD/MOD after treatment within the two treatment groups were analyzed with one-way analysis of co-variance. The interactions between the three genotypes and treatment (relative change of a certain parameter in the pravastatin group compared to the relative change of this parameter in the placebo group for the three genotypes) were tested with the interaction test of two-way covariance analysis. In case interactions between the two groups were not significant, these were also analyzed together with a correction for the effect of treatment. Additional analyses were performed in which we adjusted for age, BMI, current smoking, beta-blocker use and blood pressure.

The Cox’s regression model was used 1) to estimate the relative risk of a new clinical event between the three CYP7A1 A-278C genotypes (irrespective of treatment) and 2) to estimate the relative risk of a new event for pravastatin versus placebo treatment. Additional analyses were performed in which we adjusted for age, BMI, current smoking, beta-blocker use and blood pressure.

Cumulative event free survival was analyzed with the Kaplan-Meier method and the treatment groups were compared by the Cox regression model. Differences were considered significant at $p<0.05$. Statistical analyses were performed with SPSS software package (SPSS, Chicago, IL, USA).
RESULTS

Genotype frequencies and baseline characteristics
In total, 715 men were genotyped for the CYP7A1 A-278C polymorphism. The CYP7A1 A-278C genotype distribution was as follows: 283 subjects were homozygous for the A-allele (40%), 102 subjects homozygous for the C-allele (14%) and 330 subjects were heterozygous (46%). The observed allele frequencies were in Hardy-Weinberg equilibrium and were comparable to those in literature (4).

Patient baseline characteristics and lipid profiles according to the CYP7A1 A-278C genotype are shown in Table 5.1. There were no significant differences between the three CYP7A1 A-278C genotype groups in baseline characteristics and serum lipid levels shown in this table.

Response to treatment
Overall, upon treatment with pravastatin, levels of serum total cholesterol decreased by 20%, LDL-cholesterol by 29% and triglycerides by 7%, while the levels of HDL-cholesterol increased by 10%. Mean changes in serum lipid levels according to CYP7A1 A-278C genotype are shown in Table 5.2. The lipid-lowering effect of pravastatin on HDL- and LDL-cholesterol and triglycerides was similar in all three genotype groups. Lipid levels in the placebo group did not change markedly during the study.

Effect on progression of atherosclerosis, reflected by changes in MSD and MOD
The effect of the CYP7A1 A-278C genotype on changes in MSD and MOD is shown in Figure 5.1. Because interactions between genotype and treatment groups were not significant \( p = 0.82 \) for MSD and \( p = 0.11 \) for MOD, the treatment groups were analyzed together.

In the crude analysis, patients with the genotype CC have a significantly larger decrease in both MSD (resp. 0.09 mm versus 0.06 mm; \( p = 0.009 \)) and MOD (resp. 0.09 mm versus 0.05 mm; \( p = 0.024 \)) as compared to patients with the genotype AA, indicating more progression of diffuse and focal atherosclerosis.
In the adjusted analysis, the effect on MSD is also significant \((p=0.011)\), whereas the effect on MOD failed to reach statistical significance \((p=0.06)\). However, it should be noted that none of the risk factors we adjusted for are significantly associated with changes in MSD or MOD (all \(p\)-values>0.1).

**Clinical events**

In the pravastatin group, 13 (8%) of the 155 patients with the AA genotype had a new clinical event, compared with 16 (10%) of the 162 patients with the AC genotype and 10 (22%) of the 46 patients with the CC genotype (Figure 5.2 Pravastatin: \(p=0.027\)). After 2 years, within the pravastatin group, the relative risk of a new clinical event of patients with the genotype CC is 2.90 (95% CI 1.27-6.62; \(p=0.027\)) as compared to subjects with the genotype AA.

In the placebo group, 18 (14%) of 128 patients with the AA genotype had a new clinical event, compared with 34 (20%) of the 168 patients with the AC genotype and 11 (20%) of the 56 patients with the CC genotype (Figure 5.2 Placebo: \(p=0.39\)). In this group, there were no statistical significant differences in relative risk between the three genotype groups.

Since the interaction between genotype and treatment groups was not significant \((p=0.23)\), the treatment groups were analyzed together. Overall, irrespective of treatment, in the crude analysis, the relative risk of a new clinical event for all patients with the genotype CC is almost twice as high as compared to subjects with the genotype AA \((RR = 1.93; 95\% \ CI 1.11-3.36; p=0.02)\) (Table 5.3). In the adjusted analysis, this effect was less pronounced \((RR = 1.74; 95\% \ CI 0.96-3.12; p=0.06)\). However, it should be noted that none of the risk factors we adjusted for are significantly associated with clinical events (all \(p\)-values>0.1).

**CYP7A1 A-278C polymorphism and treatment**

We also investigated the effect of the CYP7A1 A-278C polymorphism on treatment with pravastatin versus placebo. After pravastatin treatment, the relative risk of a new clinical event was 0.59 in the AA group (95% CI 0.29-1.20; \(p=0.15\)), 0.47 in the AC group (95% CI 0.26-0.85; \(p=0.01\)) and 1.16 in the CC group (95% CI 0.49-2.73; \(p=0.74\)).
<table>
<thead>
<tr>
<th></th>
<th>AA n = 283</th>
<th>AC n = 330</th>
<th>CC n = 102</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>57 ± 8</td>
<td>56 ± 8</td>
<td>56 ± 8</td>
<td>0.17</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.9 ± 2.6</td>
<td>26.0 ± 2.7</td>
<td>26.2 ± 2.7</td>
<td>0.62</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>134 ± 18</td>
<td>136 ± 19</td>
<td>135 ± 20</td>
<td>0.19</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81 ± 11</td>
<td>82 ± 10</td>
<td>80 ± 10</td>
<td>0.09</td>
</tr>
<tr>
<td>Smoking ever (n, %)</td>
<td>247 (87%)</td>
<td>285 (86%)</td>
<td>93 (91%)</td>
<td>0.44</td>
</tr>
<tr>
<td>Smoking (n, %)</td>
<td>69 (24%)</td>
<td>94 (29%)</td>
<td>31 (30%)</td>
<td>0.38</td>
</tr>
<tr>
<td>Familial Heart Disease (n, %)</td>
<td>119 (42%)</td>
<td>168 (51%)</td>
<td>52 (51%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Myocardial Infarction (n, %)</td>
<td>141 (50%)</td>
<td>144 (44%)</td>
<td>48 (47%)</td>
<td>0.31</td>
</tr>
<tr>
<td>Hypertension (n, %)</td>
<td>76 (27%)</td>
<td>95 (29%)</td>
<td>31 (30%)</td>
<td>0.76</td>
</tr>
<tr>
<td>Pravastatin (n, %)</td>
<td>155 (55%)</td>
<td>162 (49%)</td>
<td>46 (45%)</td>
<td>0.17</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.02 ± 0.88</td>
<td>6.07 ± 0.88</td>
<td>5.93 ± 0.85</td>
<td>0.41</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>4.30 ± 0.79</td>
<td>4.32 ± 0.79</td>
<td>4.21 ± 0.79</td>
<td>0.50</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.91 ± 0.2</td>
<td>0.94 ± 0.24</td>
<td>0.91 ± 0.22</td>
<td>0.31</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.79 ± 0.77</td>
<td>1.79 ± 0.77</td>
<td>1.79 ± 0.78</td>
<td>0.99</td>
</tr>
<tr>
<td>LDL class A (n, %)</td>
<td>53 (56%)</td>
<td>72 (60%)</td>
<td>29 (73%)</td>
<td>0.46</td>
</tr>
<tr>
<td>LDL class B (n, %)</td>
<td>38 (40%)</td>
<td>42 (35%)</td>
<td>11 (27%)</td>
<td></td>
</tr>
<tr>
<td>LDL class AB (n, %)</td>
<td>4 (4%)</td>
<td>7 (5%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>MSD (mm)</td>
<td>2.75 ± 0.38</td>
<td>2.73 ± 0.37</td>
<td>2.70 ± 0.38</td>
<td>0.47</td>
</tr>
<tr>
<td>MOD (mm)</td>
<td>1.77 ± 0.38</td>
<td>1.77 ± 0.35</td>
<td>1.76 ± 0.35</td>
<td>0.93</td>
</tr>
</tbody>
</table>

- **p**-value of one-way ANOVA or Pearson chi-square test, where appropriate
- Serum lipid levels, age, BMI and blood pressure (BP) and Mean Segment Diameter (MSD) are shown as mean ± SD. Minimum Obstruction Diameter (MOD) is shown as median ± interquartile range
- Classification of LDL: Class A = predominantly large buoyant LDL; Class B = predominantly small dense LDL; Class A/B = no distinctive A- or B- pattern present
TABLE 5.2 Change of serum lipids after pravastatin and placebo treatment according to CYP7A1 A-278C genotype

<table>
<thead>
<tr>
<th></th>
<th>Placebo n = 352</th>
<th>Pravastatin n = 363</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA (n=128)</td>
<td>AC (n=168)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>56 ± 8</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>0.15 ± 0.73</td>
<td>0.12 ± 0.60</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.04 ± 0.13</td>
<td>0.04 ± 0.13</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.16 ± 0.67</td>
<td>0.12 ± 0.58</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>0.04 ± 0.65</td>
<td>0.02 ± 0.53</td>
</tr>
</tbody>
</table>

- $p$-value of one-way analysis of co-variance, with baseline values as covariates
- Changes in serum lipid levels are shown as mean ± SD
<table>
<thead>
<tr>
<th>CYP7A1 A-278C</th>
<th>Crude analysis</th>
<th>Adjusted analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR (95% CI)</td>
<td>p</td>
</tr>
<tr>
<td>AA</td>
<td>1.00 (-)</td>
<td>-</td>
</tr>
<tr>
<td>AC</td>
<td>1.37 (0.87 - 2.14)</td>
<td>0.17</td>
</tr>
<tr>
<td>CC</td>
<td>1.93 (1.11 - 3.36)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

- Relative risk (RR) is shown with 95% CI for a new cardiovascular event in the three different CYP7A1 A-278C genotype groups
- In the crude analysis, the RR was adjusted for treatment. In the adjusted analysis, the RR was adjusted for treatment, age, BMI, current smoking, beta-blocker use and blood pressure
- The AA group was taken as reference group and COX-regression analyses were performed
**FIGURE 5.1** Changes in MSD and MOD according to CYP7A1 A-278C genotype.

The delta- MSD is shown in mean ± SD, whereas the delta-MOD is shown in median ± interquartile range. Numbers: AA n= 283, AC n = 330, CC n = 102
* indicates $p < 0.05$ in the crude analysis (analysis of covariance, with effect of treatment as covariate)
** indicates $p < 0.05$ in crude and adjusted analysis (analysis of covariance, with age, BMI, current smoking, beta-blocker use, blood pressure and effect of treatment as covariates)

**FIGURE 5.2** Time-to-event analysis (Kaplan-Meier) for treatment with pravastatin and placebo, according to CYP7A1 A-278C genotype.

Treatment groups were compared by the Cox regression model.
DISCUSSION

In the present study, we investigated the association between the A-278C variant in the CYP7A1 gene and the progression of coronary atherosclerosis in a large cohort of men. The results indicate that this genetic marker is associated with the progression of coronary atherosclerosis as well as the risk of new cardiovascular events. So far, most association studies with this polymorphism only focused on variations in serum lipid levels. The effect of this polymorphism on other parameters related to the progression of atherosclerosis had not been studied in detail before.

In contrast to literature (4,5,11,12), we found no associations between this polymorphism and baseline serum lipid levels. This may be due to the fact that the REGRESS-study only included patients preselected on having total cholesterol levels between 4 and 8 mmol/l, and triglyceride levels below 4 mmol/l. Subjects in other previous studies were mainly healthy and normolipidemic, whereas our population consisted of men with angiographically documented CAD, with moderately elevated serum total- and LDL-cholesterol levels. These deviant phenotypes are probably the result of a mix of adverse genetic factors and it is conceivable that the effect of the CYP7A1 polymorphism is therefore less pronounced in our population. Furthermore, we also did not find an association between the CYP7A1 A-278C polymorphism and serum lipid reduction to pravastatin treatment. This is in contrast to a study by Kajinami et al. (13), which reported a significantly larger decrease of serum LDL-cholesterol in subjects with the genotype AA as compared to subjects with the genotype CC after atorvastatin treatment. Interestingly, in line with this difference in responsiveness between genotypes, the CYP7A1 A-278C polymorphism has recently been reported to affect the response of serum lipid levels after dietary interventions as well. Subjects with the genotype CC display a higher response of serum lipid levels after an excess dietary cholesterol or cafestol intake (14) and after a high-fat diet (15). However, the mechanism by which this polymorphism affects the response of serum lipid levels to different fatty acids or lipid-lowering drugs is as yet unknown.

The present study revealed a relation for the whole study population between the CC variant and an increased progression of atherosclerosis (as indicated by the MSD and MOD), which is known to be a predictor for the long term occurrence of clinical events (16,17). In addition, even within the two-year
duration of this study, the risk of a new clinical event in patients with the CC variant is almost twice as high as compared to patients with the AA variant. Although the inclusion of several risk factors for CHD in a multivariate model weakened our results, it is important to note that none of these factors were significantly associated with the endpoint measure. Therefore, one could question the necessity of including these factors in our model. Our findings are strengthened by a study of Couture et al. (5), which reported that, in a sample of the Framingham Offspring Study, both men and women with the CC variant had an increased history of CHD, although also this association was weakened after adjustment for CHD risk factors. However, these data possibly suggest a less favorable outcome for patients with the genotype CC and since 15% of the population carries this variant, we feel that these results are considerable.

Interestingly, after two years of treatment with pravastatin, the risk of a new clinical event seems reduced in patients with the genotype AA and AC, but not in patients with the genotype CC. Patients with the genotype CC seem to have a poorer event free survival, compared to patients with the genotype AC/AA after pravastatin treatment. It is well known that, despite the potential of cholesterol-lowering drugs like statins to reduce the incidence of CHD (6,18), these drugs do not prevent its occurrence in all patients. However, the understanding of why not all patients benefit from such therapy is still limited (19) and most probably multiple environmental and genetic factors contribute to this phenomenon. Similarly, a genetic predisposition to the response of statin medication has been described before for the REGRESS population group (7). Patients with the B2B2 genotype of the TAQ1B polymorphism in the CETP gene had the least progression of atherosclerotic lesions when treated with placebo, but had the strongest progression of lesions after statin treatment, suggesting that these subjects also have less benefit from statin treatment.

It is important to mention that the association between the CC variant and increased progression of atherosclerosis and risk of events appears independent from serum lipid levels, and is therefore more difficult to interpret. However, an effect on CHD independently from serum lipid levels has been reported before. For instance, the MTP-493G/T polymorphism is associated with a reduction in serum total cholesterol levels, but with an increased risk of CHD (20). Possibly the flux of cholesterol in and out of the circulation is as important as the absolute serum values. The underlying molecular mechanism
of the effects of the CYP7A1 A-278C polymorphism is as yet unknown. Studies on the transcriptional regulation of CYP7A1 revealed that the promoter region between nt -432 and –220 contains several cell-specific enhancer elements whose activity is controlled, in part, by HNF-3 (21). It is conceivable therefore, that the A-278C polymorphism modulates transcriptional activity of the CYP7A1 gene and, consequently, the rate of cholesterol catabolism and the flux of cholesterol in the body. The latter processes obviously are related to the progression of atherosclerosis.

In conclusion, we present evidence that the CC variant of the rate-limiting enzyme in the catabolism of cholesterol, CYP7A1, increases the progression of atherosclerosis and the risk of a new clinical event. This finding is considered to be relevant since 15% of the population carries the CC genotype.

ACKNOWLEDGEMENT
This study was financially supported by a grant from ZonMw and the Netherlands Heart Foundation (NHS) (980-10-024). The REGRESS study was sponsored by Bristol-Meyers Squibb, New Jersey, USA. JW Jukema is an established clinical investigator of the Netherlands Heart Foundation 2001 (D032).
REFERENCES


Heritable variation of serum lipid levels; no evidence for a major role of CYP7A1, CYP27 and IBAT

Hofman MK, Heijmans BT, Beekman M, Boomsma DI, Slagboom PE, Princen HMG

Manuscript in preparation
ABSTRACT

Background
One of the major pathways for eliminating cholesterol from the body is via conversion into bile acids. Genetic variations in bile acid metabolism are known to affect lipoprotein metabolism in rodents. In the present study we assessed the role of CYP7A1, CYP27 and IBAT in the heritable variation of serum lipid levels in humans.

Methods
For each gene, three microsatellite markers near the gene were selected. Two Dutch twin cohorts (n=85 dizygotic pairs, aged 13-22 and n=117 dizygotic pairs, aged 34-62) were genotyped for these markers and linkage analyses were performed. In addition, association analyses were performed with these markers and serum lipid levels, also including data from 148 Dutch monozygotic twin pairs.

Results
With the linkage analyses, only nominally significant LOD-scores were observed with markers and serum lipid levels, however, none were confirmed in both twin populations. With the association analyses, we observed a significant association in both an adolescent and an adult twin population for a marker near CYP7A1 (D8S285) and serum LDL-cholesterol levels. However, the interpretation of this finding remains to be established.

Conclusion
In conclusion, our findings do not support a major contribution of genetic variation at the CYP7A1, CYP27 and IBAT loci to serum lipid levels.
INTRODUCTION

Over the past years, research has been focused on identifying factors that determine serum cholesterol levels. Studies in twins show that 50-80% of the population variation in serum lipoprotein levels is attributable to genetic factors (1,2). Polymorphisms in genes encoding proteins involved in cholesterol metabolism can therefore be important determinants for these interindividual differences.

Catabolism of cholesterol into bile acids by the liver is an important pathway for the elimination of cholesterol from the body (3), and is initiated by two enzymes: cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27). Bile acids are secreted into the intestine, where they facilitate absorption of fat, vitamins and cholesterol. Then bile acids are actively transported back to the liver by, among others, the ileal bile acid transporter (IBAT/ASBT) (4). This cycling of bile acids between liver, gallbladder and intestine is called the enterohepatic circulation of bile acids and is essential for cholesterol homeostasis.

Genetic variations in bile acid metabolism affect cholesterol and triglyceride metabolism. For instance, patients with familial cholesterol 7α-hydroxylase deficiency display high levels of serum and hepatic cholesterol (5). Loss of CYP727 in humans causes a striking deficiency in bile acids, cerebrotendinous xanthomatosis (CTX), and an accumulation of cholesterol in virtually all tissues (6). In addition, disruption of the CYP27 gene in mice leads to a reduced bile acid synthesis and markedly increased triglyceride levels in serum (7). Furthermore, mutations in IBAT can cause Primary Bile Acid Malabsorption (PBAM), a disorder that is characterized by diarrhea, severe interruption of the enterohepatic circulation of bile acids and reduced serum cholesterol levels (8).

The above described mutations are relatively rare and thereby have a limited role in determination of lipid levels in the population at large. Therefore, it seems likely that milder mutations or polymorphisms exist, which, on a population level, influence cholesterol levels. A well known method for the localization of genes involved in a certain trait or intermediate phenotype is performing a genome scan in sibling pairs or families. Several genome scans have been performed, aiming at finding quantitative trait loci (QTLs) involved in cholesterol metabolism. With regard to bile acid metabolism, studies in
literature have been limited to the role of CYP7A1 in the heritable variation of serum lipid levels in humans. A study by Wang et al. (9) showed that the CYP7A1 locus was linked to high serum LDL-cholesterol levels (and not to low LDL-cholesterol levels) in two independent populations. Furthermore, allelic variation in CYP7A1 accounted for 15% of the total variation in serum LDL-cholesterol levels. Coon et al., on the other hand, found no evidence for linkage of LDL-cholesterol with the CYP7A1 gene in 347 families from the NHLBI Family Heart Study (10). To our knowledge, genome-wide scans described in literature, to date, have not identified bile acid metabolism-related genes as important genes contributing to the heritable variation in serum lipid levels. However, most genome scans have been performed in genetically isolated populations or patients with hyperlipidemia. Whether, on a population level, polymorphisms in CYP27 and IBAT affect cholesterol homeostasis, is unknown.

Therefore, in the present study we investigated the contribution of CYP7A1, CYP27 and IBAT to the heritable variation in serum lipid levels, by means of linkage and association analyses in Dutch monozygotic and dizygotic twins.

METHODS

Subjects
In this study, we used data from 85 dizygotic (DZ) and 61 monozygotic (MZ) adolescent Dutch twin pairs (aged 13-22 years) and 117 DZ and 87 MZ adult Dutch twin pairs (aged 34-62 years). The adolescent and adult twins were collected as separate samples. The recruitment of twins and the measurements of serum lipid and apolipoprotein levels have been described elsewhere (2). In short, the adolescent Dutch twin sample is part of a larger study in which cardiovascular risk factors were determined in adolescent twins and their parents (11,12). Addresses of twins were obtained from City Council population registries. Twins with both of their parents still living were contacted by a letter and data were collected between 1988 and 1992. The adult Dutch twin sample is also part of a larger study (13). Twins were recruited through advertisements in media and twin-specific letters and data were collected between 1992 and 1996. Informed consent was obtained from all participants.
Marker selection

In each population, segregation of parental alleles of each candidate gene was determined using microsatellite markers that co-segregated with the gene. For each gene, three markers were selected close to the gene (details in Table 6.2). The microsatellite markers D8S285, D8S1113, D8S1136 (for CYP7A1); D2S2944, D2S434, D2S1363 (for CYP27) and D13S1241, D13S779, D13S796 (for IBAT) were identified using the Marshfield linkage maps. The primer sequence and inter-marker distances are available at the Marshfield Medical Research Foundation website: (http://research.marshfieldclinic.org/genetics). The average heterozygosity for these markers was estimated at 0.79.

Genotyping

Genomic DNA was isolated from whole blood and from mouth swabs. The PCR was performed in 10 µl volumes, containing 10 ng genomic DNA, 200 µM of each dNTP, 0.073 µM of each primer, of which the forward primer was labeled with Cy5 (Amersham Pharmacia Biotech), 0.2 U TAQ Polymerase and TAQ buffer. Amplification was performed for 27 cycles of 30 sec at 94°C, 75 sec at 55°C and 15 sec at 72°C, with an initial denaturation period of 6 minutes.

The PCR-amplified DNA fragments were separated using short gel systems of the automated laser fluorescent DNA sequence analyzer ALFexpress (Amersham Pharmacia Biotech). High Resolution ReproGel (Amersham Pharmacia Biotech), which polymerizes during 10 min of exposure to UV light, was used to form the gel. The allele analysis was performed using Fragment Analyzer 1.02 (Amersham Pharmacia Biotech). To reduce genotyping errors, one known genotype was present on each gel, 10% of genotypes were performed in duplicate and each genotype was reviewed manually by two independent individuals. Unlikely recombinants indicative for genotyping errors were identified using Merlin software and removed (http://www.sph.umich.edu/csg/abecasis/Merlin/tour/error.html).

Biochemical analyses

All blood samples were taken after an overnight fasting period. Total cholesterol, HDL-cholesterol, apoB, apoA1 and triglyceride levels were
Statistical analyses

Linkage analysis
The heritability of serum lipid levels was estimated by means of linkage analysis of quantitative traits in siblings (DZ twins). Sib pair analysis for quantitative traits (e.g. cholesterol levels) is based on the co-inheritance of a trait value in combination with allele sharing at a marker locus. When the variation in the trait has a genetic origin, it is expected that sib pairs, sharing alleles identical by descent (IBD) at a marker locus close to the responsible locus, will also share the phenotype. Sib pairs who share alleles IBD are expected to resemble each other more than siblings who do not share alleles IBD, or only share a part of the markers IBD. Each individual randomly inherits two alleles of a marker: one from the father and one from the mother. Thus, a pair of siblings may have inherited either the same or different alleles. By genotyping sib pairs and their parents, it can be established whether they share no alleles IBD (IBD=0), one allele (IBD=1), or two alleles (IBD=2) for a particular marker. Under random Mendelian segregation, siblings have 25% chance to be IBD=0 and IBD=2 and 50% chance to be IBD=1. Evidence for linkage of a locus with a trait is found when more than 25% of the concordant sibs are IBD=2 and when more than 25% of the discordant sibs are IBD=0. In other words, if the locus under study is a QTL, phenotypic similarities of siblings should increase with the number of alleles they share. This estimate of a potential QTL effect can be translated into a LOD-score (10Logaritm of the odds ratio between likelihood of linkage and the likelihood of no linkage).

To test for linkage we used the variance components procedure, using the statistical software program MERLIN: (http://www.sph.umich.edu/csg/abecasis/Merlin/index.html).

Association analysis
For the association analysis, we also included data from the monozygotic twin pairs. To test for association, we used the 'Linkage Disequilibrium Analyses for Quantitative and Discrete Traits' (QTDT) procedure, which is available on the
internet: (http://www.sph.umich.edu/statgen/abecasis/qtldt/). This program allowed us to test for total association between a marker allele and a phenotype, in a variance component framework. Furthermore, this program allowed us to test for population stratification. Population stratification occurs when the allele frequency of the gene under study varies across subgroups of the population and if these subgroups also differ in their baseline risk of the disease or frequency of their phenotype. In this case, the observed association with a phenotype is not attributable to the gene, but to differences in e.g. ancestry. In the QTDT model, the total association effects can be partitioned into two components: a within family component ($\beta_w$) and a between family component ($\beta_b$). The within family association ($\beta_w$) cannot be influenced by population stratification since all tested individuals are descended from the same family, whereas the between family association ($\beta_b$) can be influenced by population stratification. By testing whether '$\beta_w = \beta_b$', we could investigate the presence of population stratification in our population.

RESULTS

The characteristics of the adolescent and adult Dutch twins are shown in Table 6.1.

**TABLE 6.1** Characteristics of the twin populations

<table>
<thead>
<tr>
<th></th>
<th>Adolescent Dutch twins</th>
<th>Adult Dutch twins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>17 ± 0.2</td>
<td>44 ± 0.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20 ± 0.2</td>
<td>25 ± 0.2</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>4.15 ± 0.05</td>
<td>5.39 ± 0.07</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.56 ± 0.05</td>
<td>3.63 ± 0.07</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.28 ± 0.02</td>
<td>1.22 ± 0.02</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.68 ± 0.02</td>
<td>1.22 ± 0.05</td>
</tr>
<tr>
<td>ApoA1 (g/l)</td>
<td>1.38 ± 0.02</td>
<td>1.67 ± 0.03</td>
</tr>
<tr>
<td>ApoB (g/l)</td>
<td>0.79 ± 0.01</td>
<td>1.21 ± 0.02</td>
</tr>
</tbody>
</table>

- Values represent means ± SEM
CHAPTER 6

Linkage

The three candidate genes were tested for linkage by variance components analysis. Table 6.2 shows the results for each marker. For ApoA1, a nominally significant LOD-score was observed with marker D13S1241 (~6 cM from the IBAT gene) in the adolescent twin population (LOD-score=1; p=0.03). However, this result was not confirmed in the adult twin population. All other LOD-scores were lower than 0.6. In addition, most of these LOD-scores are found in one twin population and are not confirmed in the other twin population.

Association

We investigated whether the alleles of the microsatellite markers were associated with serum lipid and lipoprotein levels in the adolescent and adult Dutch twin populations. For this analysis, genotypes of monozygotic twins were also included to increase power. There were no indications for population stratification. We observed a significant association in both the adolescent and the adult twin populations for marker D8S285 with LDL-cholesterol levels (p=0.007). Marker alleles with corresponding LDL-cholesterol levels are shown in Table 6.3. In both populations, allele 7 is associated with the lowest LDL-cholesterol value and allele 9 with the highest LDL-cholesterol value. We observed no other associations between the microsatellite markers and serum lipid levels that were significant in both twin populations (data not shown).

TABLE 6.3 D8S285 marker alleles and LDL-cholesterol levels

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency (%)</th>
<th>Adolescent LDL-cholesterol (mmol/l)</th>
<th>Adult LDL-cholesterol (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>16</td>
<td>2.54 ± 0.07</td>
<td>3.68 ± 0.07</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>2.46 ± 0.05</td>
<td>3.51 ± 0.07</td>
</tr>
<tr>
<td>8</td>
<td>39</td>
<td>2.67 ± 0.05</td>
<td>3.71 ± 0.06</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>2.88 ± 0.10</td>
<td>3.87 ± 0.10</td>
</tr>
<tr>
<td>Remaining alleles</td>
<td>11</td>
<td>2.55 ± 0.09</td>
<td>3.59 ± 0.13</td>
</tr>
</tbody>
</table>

- Values represent means ± SEM
- Marker alleles with a frequency of >5% are shown. Alleles with a frequency of <5% are summarized as ‘remaining alleles’ (allele 1, 3-6, 10-11)
- Variance component analysis was performed; p=0.007
<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Location (cM)</th>
<th>Adolescent twins</th>
<th>Adult twins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>LDL</td>
</tr>
<tr>
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In the present study we investigated the role of three genes important in bile acid and cholesterol homeostasis: CYP7A1, CYP27 and IBAT. We found no evidence for linkage of CYP7A1, CYP27 and IBAT with serum lipid or lipoprotein levels in two independent Dutch twin populations. This indicates that polymorphisms in these genes do not account, in a major way, for the heritable variation in serum lipid levels. However, we did observe evidence for association of a marker located in the region of the CYP7A1 locus (D8S285) with serum LDL-cholesterol levels.

A previous study by Wang et al. (9) reported evidence for linkage of CYP7A1 with high LDL-cholesterol levels in two populations: a group of 150 healthy nuclear families (934 individuals) and a group of 54 nuclear families ascertained through individuals who had premature coronary artery disease (347 individuals). Serum LDL-cholesterol levels were more highly correlated among siblings sharing both CYP7A1 alleles in common than among siblings sharing no CYP7A1 alleles. Wang et al. used four microsatellite markers in the linkage analysis, of which two were the same as in our study (D8S285 and D8S1113). However, we did not observe any evidence for linkage of CYP7A1 with LDL-cholesterol levels in two independent Dutch dizygotic twin populations: 85 adolescent twin pairs and their parents and 117 adult twin pairs. Our results are supported by a study from Coon et al. (10), which also reported no evidence for linkage of CYP7A1 (marker D8S285) with serum LDL-cholesterol levels in 347 families (939 individuals) from the NHLBI Family Heart Study. There can be several explanations for these differences in results. First of all, Wang et al. only found significant linkage of CYP7A1 with LDL-cholesterol levels after categorizing siblings in different LDL-cholesterol percentiles. CYP7A1 was significantly linked to LDL-cholesterol levels in siblings with levels exceeding the 80th percentile, and not to LDL-cholesterol levels overall. Coon et al. did not make this selection between high and low LDL-cholesterol levels. We also did not analyze specific subgroups of LDL-cholesterol levels since this would decrease the number of subjects in each group, and thereby statistical power. Other reasons for the different findings might be attributable to inter-study differences, such as ethnicity, sample size and analysis methods.

The magnitude of the potential QTL effect should also be taken in consideration. From literature, it has become clear that QTLs with only a small
impact are difficult to detect by regular linkage approaches (15). In general, significant linkage is only found when the QTL explains at least 10-20% of the heritable variation in serum lipid levels. On the basis of our linkage analysis, we thus cannot exclude that milder effects do exist for CYP7A1, CYP27 and IBAT. For this reason we decided to perform association analyses with the microsatellite markers and serum lipid levels. We observed evidence for association of a marker near the CYP7A1 locus (D8S285) with serum LDL-cholesterol levels. Interestingly, in both twin populations, similar marker alleles were associated with high and low LDL-cholesterol levels. This association might be attributable to a polymorphism in or near the CYP7A1 locus. In the promoter of the CYP7A1 gene a polymorphism has been found (the CYP7A1 A-278C polymorphism), which has been associated with LDL-cholesterol levels (9,16,17). Whether our observed association between the D8S285 marker and LDL-cholesterol levels can be attributed to the effect of the CYP7A1 A-278C polymorphism is as yet unknown. However, given the large physical distance between the CYP7A1 locus and marker D8S285 (2 million base pairs), linkage disequilibrium between these two factors is unlikely.

Until now, with respect to bile acid metabolism, QTL studies in literature have been limited to the role of CYP7A1 in the heritable variation of serum lipid levels in humans. The role of CYP27 and IBAT had not been studied before. Strategies on elucidating genetic factors involved in the variation of serum lipid levels in the general population often comprise a combined approach. First, linkage analyses are performed, either by conducting a genome scan to identify new chromosomal regions as sources of interindividual variation in a certain trait or, like we did, by investigating a specific QTL already known to be involved in the trait. Secondly, SNP’s in the DNA of the interesting area are selected either by searching public databases for previously discovered SNP’s or by sequencing the specific area to identify new informative polymorphisms. Thirdly, these informative polymorphisms are tested by genetic association studies. Based on our data, it seems not worthwhile to screen for new informative polymorphisms in CYP7A1, CYP27 and IBAT in order to investigate the variability in serum lipid levels in the general population. However, to test the role of these genes in the heritable variation of serum lipid levels with certainty, additional studies with more SNP’s in and around each gene are desired.

In conclusion, our findings do not support a major contribution of genetic variation at the CYP7A1, CYP27 and IBAT loci to serum lipid levels.
ACKNOWLEDGEMENT

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GENERAL DISCUSSION
CHAPTER 7

The aim of the studies described in this thesis was to investigate the role of variation in genes involved in bile acid metabolism on lipoprotein homeostasis in humans. Within this objective, we formulated several specific research questions. In this chapter we will first review the main findings of each research question and compare our results with literature. Hereafter, we will interpret these results and discuss critical methodological issues. Based on these considerations we will draw conclusions. Finally, we will formulate the implications of our studies and suggestions for future research.

MAIN FINDINGS

The main findings of the studies in this thesis are summarized in Table 7.1. We will now shortly address each research question, as formulated in the introduction of this thesis, and compare our results with literature.

- CYP7A1 A-278C and serum lipid levels in different populations

From epidemiological studies it was known that about 50% of the variation in serum LDL-cholesterol levels is caused by genetic factors (1,2). We hypothesized that polymorphisms in genes involved in bile acid metabolism might be important determinants of these differences. In addition, these polymorphisms might also contribute to the development of disorders in which the lipid metabolism is disturbed. As far as we know, the role of genetic variants in CYP7A1 in hyperlipidemic disorders had never been studied before. When we compared allele frequencies of the CYP7A1 A-278C polymorphism of four known hyperlipidemic disorders (HTG, CH, FD, and FH) with a normolipidemic population, we observed no differences in frequency distribution of the genotype alleles between these populations (chapter 2). In all populations, the frequency of the C-allele was approximately 40%.

The first reported association study in literature with the CYP7A1 A-278C polymorphism and serum lipid levels, described an association of the genotype CC with increased LDL-cholesterol levels (3). However, this was not confirmed consistently in later studies (4,5). When we investigated the association between the CYP7A1 A-278C polymorphism and serum lipid levels we observed an association between the genotype AA and increased serum triglyceride levels in a normolipidemic population (chapter 2).
**TABLE 7.1 Overview of main findings of this thesis**

<table>
<thead>
<tr>
<th>Ch</th>
<th>Research Question</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
</table>
| 2  | CYP7A1 A-278C and serum lipids | Association study: | • Normolipidemic: AA 34% increased TG  
• HTG (n = 139)  
• CH (n = 92)  
• FD (n=157)  
• FH (n=272)  |
| 3  | CYP7A1 A-278C and response of serum lipids | Association study:  
26 pooled dietary trials on cholesterol, saturated fat, trans fat and cafestol | • Cholesterol intake: AA lower HDL response  
• Cafestol intake: AA lower TC response |
| 4  | CYP7A1 A-278C and cholesterol-raising and cholesterol-lowering intervention (n = 94) | Response study: | • No difference in response between CC and AA in lipid and 7α-hydroxy-4-cholesten-3-one levels |
| 5  | CYP7A1 A-278C and clinical endpoints | Association study: REGRESS-study group (n=715) | • CC larger decrease in MSD and MOD  
• CC RR of clinical event = 1.93 |
| 6  | CYP7A1, CYP27 and IBAT and heritable variation in serum lipids | Linkage analyses in 202 DZ twin pairs. Association analyses in 202 DZ and 148 MZ twin pairs | • No linkage of CYP7A1, CYP27 and IBAT with serum lipids  
• Association of marker D8S285 with LDL-cholesterol levels |

HTG = Hypertriglyceridemia; CH = Combined Hyperlipidemia; FD = Familial Dysbetalipoproteinemia; FH = Familial Hypercholesterolemia; MSD = Mean Segment Diameter; MOD = Minimum Obstruction Diameter; TC=Total cholesterol; TG = Triglycerides; DZ = dizygotic; MZ = monozygotic

It is important to note that this finding was only significant in a subgroup of the population (subjects homozygous for the apoE3 allele). This observed effect on triglyceride levels had not been reported before, although Couture et al. (4) described a small difference between CC and AC carriers in women only. Unfortunately, LDL-cholesterol levels were not available for our population, which makes it difficult to compare our results to literature. However, we found no associations with serum apoB levels, an apolipoprotein which is associated with LDL particles. In patients with HTG we observed an association between...
the genotype AA and elevated serum total cholesterol levels. Furthermore, serum levels of VLDL-cholesterol, VLDL-triglycerides and triglycerides tended to be higher as well. We observed no associations between the CYP7A1 A-278C polymorphism and serum lipid levels in patients with CH, FD and FH.

- CYP7A1 A-278C and response of serum lipid levels

Besides interindividual differences in serum lipid levels, the response of serum lipid levels to diet varies as well between individuals (6). Identification of genetic factors that affect this response to diet can be helpful for selecting proper treatment for subjects with elevated serum lipid levels. Therefore, we performed a data analysis on the association between the CYP7A1 A-278C polymorphism and the response of serum lipid levels after an increased dietary intake of saturated fat, trans fat, cholesterol and cafestol (chapter 3). After an extra dietary cholesterol intake, we observed a higher response of serum total- and HDL-cholesterol levels in subjects with the genotype CC. In addition, subjects with the genotype CC also displayed a higher response of serum total cholesterol upon intake of cafestol. Our results were in line with another study, which reported that subjects with the genotype CC have a higher response of serum total cholesterol levels in an 8-year follow-up study, in a group of men of whom total cholesterol levels decreased due to a marked change in dietary composition (7).

To us, the above described data were an indication that the CYP7A1 A-278C polymorphism could play a role in an individual’s sensitivity to dietary changes. However, a limitation of our study described in chapter 3 was that results were obtained after pooling data from previously conducted interventions. We cannot exclude that this pooling might have affected the results. Furthermore, the number of subjects for some interventions was very low (e.g. for the cholesterol intervention; LDL-cholesterol levels were available for only three subjects with the genotype CC). Therefore, we decided to investigate the role of the CYP7A1 A-278C polymorphism in lipid response to a cholesterol-raising and a cholesterol-lowering intervention, in which participants were preselected for the CYP7A1 A-278C polymorphism (chapter 4). We screened a large number of subjects to obtain sufficient participants with the genotype CC, ensuring adequate statistical power. Furthermore, we measured the response of plasma 7alpha-hydroxy-4-cholesten-3-one levels, a marker that reflects the
activity of CYP7A1 and bile acid synthetic activity in the liver (8). We found no difference in response of serum lipid levels between subjects with the genotype CC and AA after a cholesterol-raising intervention (extra dietary cholesterol intake). We were thereby unable to replicate the results of our first response study. In addition, we found no difference in response of serum lipid levels between genotype groups after a cholesterol-lowering intervention (cholestyramine intake). Furthermore, plasma levels of 7α-hydroxy-4-cholesten-3-one did not differ between CYP7A1 A-278C genotypes, although response of treatment on 7α-hydroxy-4-cholesten-3-one levels was clearly present.

• CYP7A1 A-278C and clinical endpoints
Data from The Framingham Offspring Study suggested that, besides effects on serum lipid levels, the CYP7A1 A-278C polymorphism might also affect the incidence of coronary heart disease (4). Both men and women with the genotype CC had a more frequent history of coronary heart disease compared to subjects with the genotype AA. Our study in the REGRESS population confirmed these results (chapter 5). Progression of atherosclerosis, as indicated by the mean segment diameter (MSD) and minimum obstruction diameter (MOD) in the coronary artery, was significantly lower in subjects with the genotype AA. Furthermore, the relative risk of a new clinical event was almost twice as high in subjects with the genotype CC.

• CYP7A1, CYP27 and IBAT and heritable variation of serum lipids
Originally, an additional aim of this thesis research was to screen for new informative polymorphisms in other genes (besides CYP7A1) important in bile acid metabolism, for instance CYP27 and IBAT. Before starting such a screening, which would imply extensive sequencing procedures, we wanted to obtain more information about the relevance of these genes in the heritable variation of serum lipid levels. With respect to bile acid metabolism, studies in literature had so far been limited to the role of CYP7A1 in the heritable variation of serum lipid levels in humans (3,9). Therefore, we performed linkage analyses with selected microsatellite markers in and around the genes of CYP27, IBAT and CYP7A1 (the latter as a positive control) and serum lipid levels in twins and their parents (chapter 6). We observed no evidence for linkage of serum lipid levels to these three genes. Association analyses with
the microsatellite markers and serum lipid levels showed an association between marker D8S285 (near the CYP7A1 locus) and serum LDL-cholesterol levels, in two independent twin populations. However, the interpretation of this finding remains to be investigated. Our linkage results were in contradiction to the study by Wang et al., which showed that the CYP7A1 locus was linked to high serum LDL-cholesterol levels in two independent populations (3). Coon et al., on the other hand, found no evidence for linkage of LDL-cholesterol to the CYP7A1 gene in 347 families from the NHLBI Family Heart Study (9).

**INTERPRETATION OF RESULTS**

We extensively studied the CYP7A1 A-278C polymorphism in various populations (normolipidemic and hyperlipidemic) and on different endpoints (serum lipids, response of serum lipids, clinical endpoints of atherosclerosis and clinical events). In this section, we will discuss some possibilities explaining our data. First, we will suggest a molecular mechanism, explaining some (but not all) of our results. However, since we do not know whether this variant actually changes expression of the gene and thereby the rate of catabolism of cholesterol in the liver, we will discuss other options explaining our results as well.

**Molecular mechanism**

Although some of our studies showed significant results, a major drawback of these studies (and of association studies in general) is that they do not necessarily indicate a causal relationship between the genetic variant and an endpoint. Therefore, the molecular mechanism underlying the observed associations of the CYP7A1 A-278C polymorphism is difficult to pinpoint. Although we realize that our studies do not have a strong foundation to determine a possible mechanism, we believe it is interesting to speculate about it.

It is known that an increased bile acid synthesis is associated with an increase in serum triglyceride levels (10). Therefore, theoretically, the observed association between the genotype AA and elevated triglyceride levels in the normolipidemic population could be explained by an increased bile acid synthesis in subjects with the genotype AA. This is in line with the study in
which the genotype AA was associated with decreased LDL-cholesterol levels (3). If the bile acid synthesis is upregulated, more cholesterol is needed and will be taken up by the liver, leading to a decrease in serum LDL-cholesterol. For the HTG population, the AA variant could serve as an extra modulating genetic factor, next to other genetic defects, increasing triglycerides even more. Since patients with HTG have an overproduction of VLDL by the liver, containing high amounts of both triglycerides and cholesterol, the higher levels of total cholesterol and VLDL-cholesterol in patients with the genotype AA also fit into this hypothesis. Furthermore, the results of our first response study, in which subjects with the genotype AA had a smaller increase in serum cholesterol levels upon dietary cholesterol intake, are also in line with this hypothesis. An increased bile acid synthesis will, upon intake of extra dietary cholesterol, result in a more efficient removal of cholesterol from serum, leading to a decreased response. In addition, our data on the CYP7A1 A-278C polymorphism and the progression of atherosclerosis also support the association between an increased bile acid synthesis and the genotype AA. It is known that an increased bile acid synthesis is associated with a reduced risk of coronary heart disease (10) and this is what we observed for subjects with the genotype AA.

However, there is also substantial evidence that contradicts this hypothesis of an increased bile acid synthesis in subjects with the genotype AA. First of all, some studies in literature show no associations between the CYP7A1 A-278C polymorphism and serum lipid levels (5). Secondly, the hypothesis is inconsistent with the results from our second response study, which showed no difference in serum lipid levels between subjects with the genotype AA and CC after dietary changes. Finally, in our response studies and in the REGRESS-study, ‘baseline’ serum lipid levels did not differ between genotypes. If the genotype AA would result in a ‘life-long’ increased bile acid synthesis, we would expect that serum cholesterol levels would be lower in these subjects.

**Chance finding**

As is apparent from the above described section, not all our data (and data from literature) can be fit in into the same mechanism. Therefore, another possibility explaining our results might be that they are due to chance. When the number of statistical tests increases, the risk of chance finding increases as well. Especially in our first response study (chapter 3), using the pooled
dataset, we tested differences in response of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides to saturated fat, trans fat, dietary cholesterol and cafestol between genotype groups of the CYP7A1 A-278C polymorphism, with and without adjustment for several possible confounders. Thus, we performed a number of statistical tests. The probability of a spurious finding ($\alpha$) was 0.05, which means that chance finding cannot be excluded.

This issue of multiple testing is a known problem of association studies and stresses the need for replication of results in other studies that are especially designed to test the relation between serum lipid response and a candidate gene. For us, this was a major reason to set up our second response study, in which participants were preselected for the CYP7A1 A-278C genotype. The difference in results between these two response studies more so emphasizes the importance of replication of association studies, before drawing firm conclusions.

**Linkage disequilibrium**

Another possibility explaining our results is that the polymorphism is in linkage disequilibrium with another (yet unidentified or uninvestigated) mutation. In this case, it is relevant to find this mutation. Wang *et al.* (3) already described another polymorphism in the gene of CYP7A1 closely located to the A-278C variant (less than 300 base pairs in between), which was in almost complete linkage disequilibrium with the A-278C polymorphism. Whether this variant plays a functional role in the expression of the CYP7A1 gene, remains to be established.

**FUNCTIONALITY**

As mentioned before, a major limitation in extrapolating our results physiologically is that we do not know whether this genetic variant is functional. So far, no studies have been performed aiming to disentangle the functionality of the CYP7A1 A-278C polymorphism. In our second response study, we have measured plasma levels of 7alpha-hydroxy-4-cholesten-3-one and thereby used an indirect method for measuring the functionality of this genetic variant. At the beginning of the study, there were no differences in levels of 7alpha-hydroxy-4-cholesten-3-one between the CC and AA genotypes. This already
indicates that the effect, if any, of the polymorphism will not be large. Furthermore, the response of 7alpha-hydroxy-4-cholesten-3-one after the cholesterol-raising and the cholesterol-lowering intervention did not differ either.

However, by using this method, we assumed that plasma levels of 7alpha-hydroxy-4-cholesten-3-one would accurately reflect CYP7A1 activity in humans. This has been established for rats, but it has not been established in humans. Although a positive correlation exists, the precise relation between CYP7A1 activity and the amount of 7alpha-hydroxy-4-cholesten-3-one in plasma in humans is not known. It is possible that this method is not sensitive enough for detecting subtle differences in CYP7A1 activity in humans and that significant effects between genotype groups can for instance only be found in much larger sample sizes. In addition, our power calculation was based on the standard deviation of cholesterol levels and not on the standard deviation of 7alpha-hydroxy-4-cholesten-3-one. Therefore, although our results do not support a functional role for the CYP7A1 A-278C polymorphism, it still needs to be investigated.

**METHODOLOGICAL ISSUES**

Most of the methodological strengths and limitations of the research conducted in this thesis have been considered in the discussion sections of the previous chapters. Therefore, in this section, we will only focus on methodological issues that overlap in our studies and speculate whether these factors could have influenced the outcome or the choices of our studies.

**Confounding bias**

Confounding bias occurs when a potentially confounding factor that is correlated to the phenotype, is unequally matched between a group of cases and a group of controls (in case-control comparisons) or between two genotype groups (11). Possible confounders of the relationship between genetic polymorphisms and serum lipid levels are body mass index, sex and age. In our association studies we have tried to overcome this problem by including these known confounders as co-variables in a regression model. In all our studies, inclusion of these factors in the model, did not materially affect
our data. Although confounding in our studies might have occurred by variables we did not measure, we assume that due to Mendelian randomization, such unmeasured variables will have been equally divided between genotype groups.

**Selection bias**

Selection bias occurs when two groups differ in measured or unmeasured baseline characteristics because of the way participants were selected (11). In chapter 2, where we compared allele frequencies of the CYP7A1 A-278C polymorphism between different hyperlipidemic populations and a normolipidemic population, selection bias might be a critical issue. The normolipidemic population comprises men only, all 35 years of age, whereas the hyperlipidemic populations included both men and women, of various ages. Furthermore, samples from the normolipidemic population were randomly selected from three different areas in the Netherlands by means of advertisements, whereas samples from the hyperlipidemic populations were collected in a hospital after referral by a family doctor. We thus realize that our control group would not have been appropriate for the usual case-control study. However, the observed allele frequencies in our control population (and in the hyperlipidemic populations) were in line with allele frequencies in several other normolipidemic populations studied in literature (3-5). For this reason we think that this comparison was an acceptable approach and that it is safe to conclude that 1) the CYP7A1 A-278C genotype distributions are similar in all studied populations, and 2) this genetic variant is probably not associated with these disorders.

**Publication bias**

A problem that has aroused from the use of association studies is publication bias (12). In general, ‘positive’ associations are much more likely to be accepted for publication than ‘negative’ associations, and therefore the effects described in literature might be overestimated. It is possible that the CYP7A1 A-278C polymorphism has been studied in more detail by other groups, but that results have never or will never be published. The rationale for the studies described in this thesis was based on several indications from literature. First of all, animal studies showed an association between genetic factors in bile acid metabolism and serum lipid levels (13-15). Human studies indicated that
rare mutations in genes in bile acid metabolism had important phenotypic consequences for cholesterol homeostasis (16). But our rationale was also, to a considerable extent, based on the first combined linkage/association study described by Wang et al. (3), which described that genetic variation in CYP7A1 accounted for 15% to the heritable variation in serum LDL-cholesterol levels. This combination of evidence made us decide to extensively study the CYP7A1 A-278C polymorphism. However, due to the possible presence of publication bias, we cannot exclude that we might have overestimated the size of effect of the CYP7A1 A-278C polymorphism or the overall impact of genetic variation in bile acid metabolism.

**IN CONCLUSION**

Taking together our results, available information from literature and the strengths and limitations of our studies, we draw the following conclusions.

Since we found that allele frequencies of the CYP7A1 A-278C polymorphism did not differ between patient groups (HTG, CH, FD and FH) and a normolipidemic control population, we conclude that this genetic variant is not involved in the development of these disorders. Furthermore, the CYP7A1 A-278C polymorphism is inconsistently associated with serum lipid levels. It is therefore difficult to explain our results physiologically, which makes the relevance of this polymorphism in maintaining cholesterol balance in humans questionable. In addition, the CYP7A1 A-278C polymorphism is probably not involved in the response of serum lipid levels to different diets and therefore this variant will not be a useful tool to identify subjects who will benefit from dietary guidelines. Furthermore, we did not find evidence that the polymorphism leads to a functional change in enzyme activity. It is difficult to draw conclusions for the CYP7A1 A-278C polymorphism with respect to its effects on clinical endpoints of atherosclerosis. Although we observed a considerable impact on the progression of atherosclerosis and the risk of a clinical event, results need to be replicated in another study and a mechanistic basis needs to be found. Finally, the data from our linkage study indicate that the impact of genetic variants in CYP7A1, CYP27 and IBAT in the heritable variation of serum lipid levels is probably low. Therefore, it is probably not worthwhile to screen for new informative polymorphisms in these genes. The results of our association studies with the CYP7A1 A-278C polymorphism
confirm this lack of an important role of polymorphisms in bile acid metabolism on cholesterol homeostasis.

At the start of this project, the field of genetic variation and cholesterol metabolism seemed promising. However, in recent years it has become clear that cholesterol levels are not determined by one polymorphism, but presumably by lifestyle factors together with several interacting genes. Furthermore, cholesterol homeostasis is maintained by the action of several pathways, including cholesterol absorption in the intestine, cholesterol uptake by tissues and the liver, endogenous cholesterol synthesis and by the focus of this thesis: cholesterol catabolism into bile acids. Therefore, in fact it is no wonder that the effect of one single polymorphism in one gene important in bile acid metabolism is relatively modest.

**IMPLICATIONS**

The primary objective of the studies described in this thesis was to gain insight into the role of genetic variants in bile acid metabolism, in order to contribute to new insights in lipoprotein metabolism. Therefore, these studies were not designed to provide guidelines or formulate recommendations that can be used in clinical practice. However, although the results of our studies indicate no or very limited effects of the CYP7A1 A-278C polymorphism, we feel that it is still interesting to speculate about a role of this polymorphism in clinical practice.

The aim of the studies described in chapter 3 and 4 was to investigate the role of the CYP7A1 A-278C polymorphism in lipid response after different diets. If we would have found strong effects of this variant on lipid response, the polymorphism might have been a good tool to identify subjects who would benefit from dietary guidelines. The polymorphism could then possibly be implemented in clinical practice as a genetic test to predict an individual's response to diet.

Besides a test that predicts the response of serum lipids, it might also be useful to have a test that predicts genetic susceptibility for the development of cardiovascular disease. So far, the investigation of a possible heritable component influencing the development of cardiovascular disease is limited to the question of the presence of a family history in cardiovascular disease. Knowledge about genetic variants that influence cholesterol levels could be
helpful in this risk prediction. However, besides the apoE genotype, no genetic variants have been described that have been consistently associated with serum cholesterol levels and the risk for cardiovascular disease in the general population. In addition, the studies described in this thesis also indicate very limited effects of polymorphisms in bile acid metabolism.

Humphries et al. (17) postulated that, for a genetic test to be useful in clinical management of cardiovascular disease, it is critical that the test must have additional predictive power over and above those accepted risk factors that can easily be measured. In this case, even the apoE genotype is not a good tool to predict cardiovascular disease risk, since the apoE variants have the majority of their effects on risk through their effects on serum lipid levels. Incorporating lipid levels in the risk calculation will remove much (if not all) of the risk information that could be obtained from an individual’s apoE genotype. In this respect, the observed association between the CYP7A1 A-278C polymorphism and the development and progression of atherosclerosis and risk of a clinical event seems interesting, since it appears to be independent from lipid levels. However, as mentioned before, these results need to be confirmed in other populations. At this time, it is too soon to decide whether assessment of the CYP7A1 A-278C polymorphism may be implemented in clinical practice as a tool of risk prediction of cardiovascular disease.

FUTURE DIRECTIONS

- The functionality of the CYP7A1 A-278C polymorphism should be established, to find out whether this genetic variant actually affects protein expression. This can be investigated in vitro by generating promoter-luciferase constructs specific for each genotype, transfecting them into a liver cell line and measure the activity of each construct.

- The results of our association study with the CYP7A1 A-278C polymorphism on clinical endpoints need to be confirmed in another population. Furthermore, since results seem to be independent from serum lipid levels, the mechanism behind these effects should be clarified. In addition, if the association between the genotype CC and a higher risk of a clinical event is confirmed, it would be interesting to study the allele...
frequency of the CYP7A1 A-278C genotype in a population of subjects aged 85 years or older.

- As is apparent from this thesis, the effect of one polymorphism in a single gene will be relatively small in predicting cholesterol levels. Probably multiple polymorphisms act together. Therefore, simultaneous screening of several polymorphisms that have proven functionality may be a more fruitful approach. In this respect the so called 'gene micro arrays' (chips) can be very helpful in effectively approach future genetic research. Of course we realize that there are practical limitations to this approach and that success will depend on the availability of large sample size.
REFERENCES


CHAPTER 7


INTRODUCTION

An important pathway for eliminating cholesterol from the body is via the bile, either directly or after conversion into bile acids. Accordingly, therapeutic compounds that increase bile acid synthesis have been shown to reduce serum LDL-cholesterol levels and the risk of coronary heart disease. Data from literature indicate that genetic variation in bile acid metabolism has important consequences for lipidemic phenotype. The aim of the studies in this thesis was to achieve a better understanding of genetic factors in bile acid metabolism and the implications for lipoprotein homeostasis in humans.

RESULTS OF STUDIES DESCRIBED IN THIS THESIS

• CYP7A1 A-278C and serum lipid levels

Since CYP7A1 is the rate-limiting enzyme in the catabolism of cholesterol, we hypothesized that polymorphisms in the CYP7A1 gene could have important consequences for lipid metabolism in healthy individuals as well as in patients with lipid disorders. In chapter 2 we investigated a possible role of the CYP7A1 A-278C polymorphism in four known hyperlipidemic disorders: hypertriglyceridemia (HTG), combined hyperlipidemia (CH), familial dysbetalipoproteinemia (FD) and familial hypercholesterolemia (FH), by comparing allele frequencies of this polymorphism between patients and normolipidemic controls. Furthermore, we investigated the association of this polymorphism with serum lipid levels within these patient and control populations. We found no differences in the frequency distribution of the CYP7A1 A-278C alleles between the hyperlipidemic populations and a normolipidemic population, strongly indicating that the CYP7A1 A-278C polymorphism is not involved in the development of these disorders.

In the normolipidemic population, homozygous for the apoE3 allele, the genotype AA was associated with a significant 34% increase in serum triglyceride levels, as compared to the genotype CC. In patients with HTG, the genotype AA was associated with a significant 23% increase in serum total cholesterol. Furthermore, there was a trend towards increased levels of triglycerides, VLDL-cholesterol and VLDL-triglycerides in subjects with the genotype AA. We observed no associations between the CYP7A1 A-278C polymorphism and serum lipid levels in patients with CH, FD and FH.
**CYP7A1 A-278C and response of serum lipid levels**

From literature it is known that there is a large interindividual variability in the response of serum lipid levels to diet. Identification of genetic factors that affect this response to diet can be helpful for selecting proper treatment for subjects with elevated serum lipid levels. Therefore, in chapter 3, we performed a data analysis on the association between the CYP7A1 A-278C polymorphism and the response of serum lipid levels after an increased dietary intake of saturated fat, *trans* fat, cholesterol and cafestol. To this end, data of 26 previously published trials were pooled. After a cholesterol-rich diet, the genotype AA was associated with a significantly smaller increase in serum HDL-cholesterol, as compared to the genotype CC (0.00 ± 0.02 vs. 0.17 ± 0.04 mmol/l, respectively). Upon intake of cafestol, the genotype AA was associated with a significantly smaller increase in serum total cholesterol, as compared to the genotype CC (0.69 ± 0.10 vs. 1.01 ± 0.10 mmol/l, respectively). No differences were found for the saturated fat and *trans* fat interventions.

A limitation of this study was that results were obtained after pooling data from previously conducted interventions. We cannot exclude that this pooling might have affected the results. Furthermore, the number of subjects for some interventions was very low (e.g. for the cholesterol intervention; LDL-cholesterol levels were available for only three subjects with the genotype CC). Therefore, we decided to investigate the involvement of the CYP7A1 A-278C polymorphism in serum lipid response to diet in a study design which was specifically set up to study this role. Therefore, in chapter 4, we studied the role of the CYP7A1 A-278C polymorphism in the response of serum lipid levels after a cholesterol-raising and a cholesterol-lowering intervention, in a group of 94 healthy individuals with either the AA or CC genotype of this genetic variant. Furthermore, we investigated the functionality of the polymorphism by evaluating the response of plasma 7alpha-hydroxy-4-cholesten-3-one levels, a marker that reflects the activity of CYP7A1 and bile acid synthesis in the liver. There were no significant differences in the response of serum total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride levels between subjects with the genotype AA and subjects with the genotype CC after a cholesterol-raising intervention (extra dietary cholesterol intake), nor after a cholesterol-lowering intervention (cholestyramine intake). Furthermore, plasma levels and response of 7α-hydroxy-4-cholesten-3-one did not differ between CYP7A1 A-278C genotypes, indicating that this polymorphism does not result
in differences in enzyme expression. Since this study was specifically designed to study the role of the CYP7A1 A-278C polymorphism we conclude that it has no impact on serum lipid response and that there is no reason to consider screening for this genetic variant in clinical practice.

• CYP7A1 A-278C and clinical endpoints

In chapter 5, we investigated the association between the CYP7A1 A-278C polymorphism and markers of atherosclerosis. We used data from the REGRESS-study, which is a double-blind placebo controlled study to assess the effects of 2 years of treatment with pravastatin on the progression and regression of angiographically documented coronary atherosclerosis in male patients with serum cholesterol between 4 and 8 mmol/l. The progression of atherosclerosis, as indicated by the mean segment diameter (MSD) and minimum obstruction diameter (MOD) in the coronary artery was significantly lower in subjects with the genotype AA (33% and 44%, respectively) as compared to subjects with the genotype CC. Inclusion of risk factors for CHD in the model showed the same trend, although not significant for MOD (p=0.01 for MSD and p=0.06 for MOD). Furthermore, the relative risk of a new clinical event was almost twice as high in subjects with the genotype CC (RR = 1.93; 95% CI 1.11-3.36; p=0.02). Inclusion of risk factors for CHD in the model showed the same trend, although not significant (RR=1.74; 95%CI 0.96-3.12; p=0.06). These data indicate a rather large impact of the CYP7A1 A-278C polymorphism on clinical development of atherosclerosis. However, before drawing conclusions, these results should be replicated in another study.

• CYP7A1, CYP27 and IBAT and heritable variation of serum lipids

In chapter 6, we assessed the contribution of CYP7A1, CYP27 and IBAT, three key-proteins in bile acid metabolism, to the heritable variation in serum lipid levels. For each gene, we selected three microsatellite polymorphisms in and around the gene and genotyped two dizygotic Dutch twin populations (85 adolescent twin pairs and 117 adult twin pairs) and their parents for each marker and we performed both linkage and association analysis. We observed no evidence for linkage of serum lipid levels to CYP7A1, CYP27 or IBAT, in the two independent Dutch twin populations. When we performed association analysis, which also included data from 61 monozygotic adolescent twin pairs and 87 monozygotic adult twin pairs, we observed a significant association
between serum LDL-cholesterol levels and a microsatellite marker near the CYP7A1 locus (D8S285) in both the adolescent and the adult twin populations. Whether this effect is attributable to the CYP7A1 A-278C polymorphism remains to be established. In conclusion, these data indicate that polymorphisms in CYP7A1, CYP27 and IBAT probably will not affect the heritable variation in serum lipid levels to a large extent.

GENERAL CONCLUSION

In chapter 7 we have tried to interpret our studies by discussing a possible mechanism and other options explaining our results. Furthermore, we have placed our studies in perspective by reflecting on the strengths and limitations of our studies. Based on these considerations, we draw the following conclusions. First of all, the effect of the CYP7A1 A-278C polymorphism on serum lipid levels, if present, is small. In addition, the CYP7A1 A-278C polymorphism is not involved in the response of serum lipid levels after different dietary interventions. The impact of the CYP7A1 A-278C polymorphism on the development of atherosclerosis seems considerable; however, this needs to be replicated in another population before drawing firm conclusions. Finally, although we cannot exclude that other, yet uninvestigated or unidentified, polymorphisms related to bile acid metabolism have a larger effect on lipid metabolism, the size of the effect of any single variant in one gene on an intermediate phenotype related to coronary heart disease will probably be very low.
SAMENVATTING
INLEIDING

De cholesterol balans


Twee enzymen spelen een belangrijke rol bij de afbraak van cholesterol naar galzuren: "cholesterol 7alpha-hydroxylase" (CYP7A1) en "sterol 27-hydroxylase" (CYP27). De gevormde galzuren worden getransporteerd naar de darm, waar ze belangrijk zijn bij de opname van vetten, vitaminen en cholesterol uit het voedsel. Het overgrote deel van de galzuren wordt hierna weer terugtransporteerd naar de lever. Hierbij speelt het eiwit "intestinal bile acid transporter" (IBAT) een belangrijke rol. Deze zogenaamde circulatie van galzuren in het lichaam is essentieel voor het behouden van een goede balans van cholesterol in het lichaam.

Doel van de studies in dit proefschrift

Uit de literatuur is bekend dat erfelijke factoren een belangrijke rol spelen bij de balans van cholesterol in het lichaam. Het doel van dit proefschrift was het onderzoeken van de relatie tussen erfelijke factoren in het galzuurmetabolisme en de balans van cholesterol in het lichaam.

AANPAK EN RESULTATEN VAN DIT PROEFSCHRIFT

- Polymorfisme in CYP7A1 en relatie met bloed lipiden

CYP7A1 is de snelheidsbepalende stap in de afbraak van cholesterol. Een verstoorde functie van CYP7A1 kan dus leiden tot een verhoogde cholesterolconcentratie in het bloed. In het gen van CYP7A1 is een mutatie
SAMENVATTING

(ofwel polymorfisme) gevonden: het CYP7A1 A-278C polymorfisme. Dit polymorfisme komt voor bij ongeveer 15% van de bevolking. In hoofdstuk 2 is de mogelijke rol van dit polymorfisme bestudeerd bij het ontstaan van een verstoorde vetstofwisseling. Dit is bekeken in patiënten met de volgende aandoeningen: hypertriglycerideremie (HTG), gecombineerde hyperlipidemie (CH), familiare hypercholesterolemie (FH) en familiare dysbetaalipoproteïnemie (FD). Verder is in deze patiënten de relatie tussen het CYP7A1 A-278C genotype en bloed lipiden bestudeerd. Er is geen verschil gevonden in de genotype verdeling van het CYP7A1 A-278C polymorfisme van de patiënten populaties en die van een ‘gezonde’ controlepopulatie. Dit is een sterke aanwijzing dat het CYP7A1 A-278C polymorfisme geen rol speelt in de ontwikkeling van deze verschillende ziektebeelden. In de controlepopulatie was in personen met het genotype AA de triglyceride concentratie significant verhoogd (34%), vergeleken met personen met het genotype CC. In patiënten met HTG was in personen met het genotype AA het totaal cholesterol significant verhoogd (23%), vergeleken met patiënten met het genotype AC/CC. Verder was er een duidelijke, maar niet significante, relatie tussen verhoogde concentraties van triglyceride, VLDL-cholesterol en VLDL-triglyceriden in patiënten met het genotype AA, ten opzichte van patiënten met het genotype CC. Dit polymorfisme zou dus mogelijk kunnen bij dragen aan het fenotype in patiënten met HTG. Er is geen verband gevonden tussen het CYP7A1 A-278C polymorfisme en bloed lipiden in patiënten met CH, FH en FD.

- Polymorfisme in CYP7A1 en relatie met de respons van bloed lipiden

Mensen met een verhoogd cholesterol krijgen vaak het advies om een cholesterolverlagend dieet te volgen. Dit is effectief voor veel mensen maar niet voor iedereen. Andersom, zijn er mensen die veel cholesterol via hun voeding binnen krijgen zonder dat het cholesterol in het bloed stijgt. Wanneer erfelijke factoren die gerelateerd zijn aan de respons van cholesterol bekend zijn, kan dit helpen mensen te identificeren die wel of niet gebaat zullen zijn bij een bepaald dieet. Omdat CYP7A1 een belangrijke rol speelt in het cholesterol metabolisme, is in hoofdstuk 3 de relatie tussen het CYP7A1 A-278C polymorfisme en de respons van bloed lipiden bestudeerd na een verhoogde inname van verzadigd vet, trans vet, cholesterol en cafestol. Hiervoor is een dataset gebruikt waarin de resultaten van 26 voedingsproeven zijn gecombineerd. Na een cholesterolrijk dieet was de respons van HDL-
cholesterol lager in mensen met het genotype AA, vergeleken met mensen met het genotype CC (0.00 ± 0.02 vs. 0.17 ± 0.04 mmol/l). Na inname van cafestol, was de respons van totaal cholesterol lager in mensen met het genotype AA, vergeleken met mensen met het genotype CC (0.69 ± 0.10 vs. 1.01 ± 0.10 mmol/l). Er is geen relatie gevonden tussen het CYP7A1 A-278C polymorfisme en de respons van bloed lipiden na inname van verzadigd vet en trans vet.

De bovenstaande resultaten waren verkregen na het combineren van gegevens uit verschillende studies. Het is echter niet helemaal uit te sluiten dat dit vertekening van de resultaten heeft gegeven. Verder was het aantal personen bij sommige interventies erg laag (bijvoorbeeld bij de cholesterol interventie; LDL-cholesterol waarden waren beschikbaar voor slechts drie mensen met het genotype CC). Daarom is in hoofdstuk 4 de impact van het CYP7A1 A-278C polymorfisme op de respons van bloed lipiden opnieuw en meer nauwkeurig bestudeerd met behulp van een cholesterolverhogende en een cholesterolverlagende interventie, waarbij de deelnemers vooraf geselecteerd zijn op het CYP7A1 A-278C genotype. Er zijn 94 gezonde vrijwilligers geselecteerd met ofwel het AA ofwel het CC genotype. Ook is de functionaliteit van het polymorfisme geëvalueerd, door in het bloed de concentratie 7alpha-hydroxy-4-cholesten-3-one te meten. Dit is een marker die mogelijk de activiteit van CYP7A1 in de lever en de galzuursynthese snelheid weergeeft. Er was geen verschil in de respons van bloed lipiden tussen mensen met het genotype AA en mensen met het genotype CC na een cholesterolverhogende interventie (extra cholesterol inname) noch na een cholesterolverlagende interventie (cholestryramine inname). Ook was er geen verschil tussen de genotypes in de beginwaarde en de respons van de concentratie 7alpha-hydroxy-4-cholesten-3-one, wat suggereert dat het polymorfisme geen functionele verandering in de CYP7A1 enzymactiviteit veroorzaakt. Uit deze resultaten kan geconcludeerd worden dat het CYP7A1 A-278C polymorfisme de lipiden respons niet modificert en dat dit polymorfisme dus niet mensen identificeert die wel of niet zullen reageren op een cholesterolverlagende voeding.
• Polymorfisme in CYP7A1 en relatie met atherosclerose

In hoofdstuk 5 is de associatie tussen het CYP7A1 A-278C polymorfisme en markers voor atherosclerose beschreven. Hiertoe zijn data van de REGRESS-studie gebruikt. De REGRESS-studie is opgezet om het effect te onderzoeken van twee jaar behandeling met pravastatine op de progressie en regressie van angiografisch aangetoonde coronaire atherosclerose in mannelijke patiënten met een normaal tot matig verhoogd cholesterolgehalte (4-8 mmol/l). Twee maten voor de progressie van atherosclerose zijn: de gemiddelde segmentdiameter (MSD) en de minimale obstructie diameter (MOD). In patiënten met het genotype AA was de reductie van de MSD en MOD significant geringer dan in patiënten met het genotype CC, wat aangeeft dat patiënten met het genotype CC een verhoogde progressie van coronaire atherosclerose hebben. Verder hebben patiënten met het genotype CC een bijna twee keer zo hoog risico op een nieuwe complicatie vergeleken met patiënten met het genotype AA (RR = 1.93; 95% CI 1.11-3.36; p=0.02).

• Rol van de genen CYP7A1, CYP27 en IBAT

Om de bijdrage van CYP7A1, CYP27 en IBAT aan de erfelijke variatie in bloed lipiden te bestuderen, hebben we een koppelingsanalyse (linkage analyse) uitgevoerd in 85 adolescente twee-eiige tweelingen en 117 volwassen twee-eiige tweelingen (hoofdstuk 6). Rondom elk van de drie genen zijn drie DNA markers geselecteerd, waarvan de lengte is bepaald in de tweelingen populaties. Wanneer een tweeling een genetische marker van dezelfde lengte heeft, dan is met grote zekerheid te zeggen dat ze dat stukje DNA van dezelfde ouder hebben geërfd. Als nu een bepaald gen zorgt voor variatie in bijvoorbeeld cholesterol concentratie, dan zullen de tweelingen die dezelfde variant van het DNA geërfd hebben van hun ouders een meer gelijk cholesterol niveau hebben dan wanneer ze een verschillende variant geërfd hebben. Door dit in alle tweelingparen te onderzoeken, kan dus de waarschijnlijkheid (uitgedrukt in een LOD-score) worden bepaald dat een gen het bloed cholesterol niveau beïnvloedt. Er is geen sterk bewijs gevonden voor koppeling tussen de locaties van de CYP7A1, CYP27 en IBAT genen en bloed lipide concentraties. Dit suggereert dat de kans klein is dat er in één van de drie genen zich polymorfismen bevinden die van grote invloed zijn op de variatie in bloed lipide niveaus. Subtiele effecten kunnen met deze studie echter niet worden uitgesloten. Vervolgens zijn er associatie analyses uitgevoerd met de DNA markers, door te bepalen of één van de varianten van
een marker van invloed is op een bepaald lipide niveau. Hierbij zijn ook gegevens gebruikt van 61 eeneiige adolescente tweeling paren en 87 eeneiige volwassen tweeling paren. De enige significante associatie die gevonden werd in zowel de adolescente als de volwassen tweeling populatie, was de associatie tussen LDL-cholesterol concentratie en een DNA marker vlakbij het CYP7A1 gen (marker D8S285). Het is op dit moment niet duidelijk of dit effect is toe te schrijven aan het effect van het CYP7A1 A-278C polymorfisme.

CONCLUSIES

In hoofdstuk 7 hebben we de sterke en zwakke punten van de hierboven beschreven onderzoeken bediscussieerd en geprobeerd om onze resultaten te interpreteren. Op basis hiervan hebben we de volgende conclusies getrokken. De invloed van het CYP7A1 A-278C polymorfisme op de cholesterol balans in het bloed is, indien al aanwezig, niet groot. Het polymorfisme heeft bovendien ook geen effect op de respons van bloed lipiden na verschillende interventies die de cholesterolconcentratie beïnvloeden. Het polymorfisme lijkt een behoorlijk effect te hebben op de ontwikkeling van atherosclerose, maar voordat hier conclusies aan verbonden kunnen worden, moeten deze resultaten eerst bevestigd worden in een andere studie.

Tot slot, ook al kunnen we niet uitsluiten dat er andere polymorfismen in het galzuurmetabolisme een groter effect zullen hebben, lijkt het effect van één genetische variatie in één gen op de cholesterol balans in het lichaam klein.
DANKWOORD
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Maaike
ABOUT THE AUTHOR

Maaike Hofman was born on the 5th of June 1978, in Rotterdam, The Netherlands. After completing secondary school in 1996 (VWO) at the ‘CSG Blaise Pascal’ in Spijkenisse, she started the study ‘Medical Biology’ at the University of Amsterdam. As part of this study, she started her first research project at Solvay Pharmaceuticals on developing a model in rats to screen for new drugs against panic attacks. Within the framework of her specialization in Social Biology, in 2000, she started a second research project at SWOKA, on surveying the network around genetic testing and functional foods in The Netherlands. At the end of the same year she received her MSc degree. She directly started working as a PhD-fellow at the Division of Human Nutrition at the Wageningen University and the Department of Vascular and Metabolic Diseases at TNO Prevention and Health in Leiden. The project was entitled ‘Genetic variation in bile acid metabolism and the response to dietary fat’, of which the main results are presented in this thesis. She joined several courses and conferences on nutrition, epidemiology and health, within the framework of the educational program of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences). She was a member of the committee for temporary scientific staff within the Division of Human Nutrition as well as a member of the organization committee of a PhD-study tour to Australia in 2003.
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TRAINING AND SUPERVISION PLAN

Symposia and congresses

2004  Congress: Drugs Affecting Lipid Metabolism  
Venice, Italy

2003  Symposium: Future perspectives of human health;  
the role for nutrigenomics  
Wageningen, The Netherlands

2002  Congress: 73rd European Atherosclerosis Society  
Salzburg, Austria

2002  Masterclass: From nutrigenomics to healthy foods  
Maastricht, The Netherlands

2002  Congress: International Bile Acid meeting:  
Bile acids: from Genomics to Disease and Therapy  
Freiburg, Germany

2001  Congress: 72nd European Atherosclerosis Society  
Glasgow, United Kingdom

2000  International Bile Acid meeting:  
Biology of Bile Acids in Health and Disease  
The Hague, The Netherlands

2000-2004  NWO  
- Meeting of Nutritional Science Community  
- Symposium Nutrition and Chronic Diseases

Courses

2004  Functioning successfully within organizations  
VLAG course

2001  Erasmus Summer Program 2001  
- Principles of Research in Medicine and Epidemiology  
- Genetic Epidemiology  
- Genetics of Complex Diseases

General

2003  PhD study tour, Australia
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